Evaluierung Blut-und Gewebebasierter Biomarker beim Ovarialkarzinom

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Abkürzungsverzeichnis

ADCC	Antibody dependent cellular cytotoxicity
ADP	Adenosindiphosphat
AKT	Proteinkinase B
ALDH	Aldehyd-Dehydrogenase
AR	Androgenreceptor
bDNA	branched Desoxyribonukleinsäure
BRCA	Breast Cancer
CALCA	Calcitonin related Polypeptid alpha
CA-125	Cancer Antigen-125
CK	Cytokeratin
CSF	Colony Stimulating Factor
CTC	Circulating Tumor Cell
ctDNA	circulating tumor Desoxyribonucleic acid
DAPI	4´,6-diamidino-2-phenylindole
DC	Dendritic Cells
DC-CK	Dendritic cell specific CC-Chemokine
DTC	Disseminated Tumor Cell
DNA	Desoxyribonucleic acid
DEP	Dielectrophoretisis
DEPP-FFF	Dielectrophoretisis field flow fractionation
DTZ	Disseminierte Tumorzellen
EGFR	Endothelial Growth-Factor-Receptor
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicine Agency

EMT	Epithelial-Mesenchymal Transition
EP300	Histone-Acetyltransferase p300
EpCAM	Epithelial Cell Adhesion Molecule
ERCC	Excision-Repair Cross-Complementation
ERK	Extracellular-signal Regulated Kinase FC-Region Fragment-crystallisable Region
FDA	Food and Drug Administration
FIGO	Fédération Internationale de Gynécologie et d´Obstétrique
FLT	Fms-Like Tyrosinkinase Receptor
GlycoA	Glycophorin A
GM-CSF	Granulocyte-Monocyte-Colony-Stimulating Factor
GSK	Glycogen Synthase Kinase
HEA-125	Human Epithelial Antigen 125
HER	Humane Epidermal Growth Factor Receptor
ICC	Immuncytochemie
IFN	Interferon
IL	Interleukin
ISET	Isolation by Size of Epithelial Tumor Cells
KM	Knochenmark
LNCAP	Androgen sensitive humane Prostata Adenokarzinoma Cell line
LOH	Loss of Heterozygoity
MEK	Mitogen-Activated Protein Kinase
MET	Mesenchymale-Epitheliale Transition
MOFF	Multi-Orifice Flow Fractionation

mRNA	messenger Ribonucleic Acid
miRNA	micro Ribonucleic Acid
MS-HRMA	Methylation-sensitive High-Resolution Melting Analysis
MSP-PCR	Methylation-Specific Polymerase Chain-Reaction
MUC	Mucin
Neg	Negative
Ng	Nanogramm
Nt	Nucleotide
NK	Natural Killer Cells
OS	Overall survival
OCT	Octamer binding Transcriptions Factor
PARP	Poly-ADP-Ribose-Polymerase
PCNA	Proliferating-Cell-Nuclear-Antigen
PDGF	Platted Derived Growth Factor
PFS	Progression free survival
PiRNA	Piwi-interacting RNA
PI3K(α)	Phosphoinositid 3-Kinase(α)
Pos	Positive
PSA	Prostate Specific Antigen
PSMA	Prostate Specific Membrane Antigen
qPCR	Quantitative Polymerase Chain Reaction
RAF	Rapidly Acclerated Fibrosarcoma
RAN-GTP	Ras related Nuclear Protein-Guanosintriphosphat
RASSF-1	RAS-Association Domain-Containing Protein 1
RISC	RNA-Induced-Silencing-Complex

RBC	Red blood cells
RFC	Replication Factor C
RPA	Replication Protein A
TFS-2	Transcription Elongation Factor S 2
TFIIH	Transcription Factor IIH
TNF	Tumor Necrosis Factor
T-RNA	Transfer RNA
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
Vs	Versus
XPA	Xeroderma Pigmentosum Complementation Gruppe A
XPC	Xeroderma Pigmentosum Complementation Gruppe C
XPG	Xeroderma Pigmentosum Complementation Gruppe G
XPF	Xeroderma Pigmentosum Complementation Gruppe F
Y-RNA	Y-Ribonucleic Acid
zfDNA	zirkulierende freie Tumor Desoxyribonukleinsäure
zirmiRNA	Zirkulierende Mikroribonukleinsäure
ztDNA	zirkulierende Tumor Desoxyribonukleinsäure
ZTZ	Zirkulierende Tumorzellen

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1 Zusammenfassung

Trotz fortgeschrittener Behandlungsmöglichkeiten beträgt die Rezidivrate beim Ovarialkarzinom etwa 75%, wobei 15-20% der Patientinnen platinresistent sind, was bisher nur retrospektiv erkannt wird. Somit ist dem Ovarialkarzinom eine sehr schlechte Prognose zuzuordnen und es ist von großem Interesse, innovative, blutbasierte Biomarkerkonzepte zu entwickeln, die uns Aufschluss über Prognose, Rezidiventwicklung, Therapieansprechen geben oder im Therapieverlauf genutzt werden können.

Im Rahmen dieser Doktorarbeit wurden zirkulierende Tumorzellen im Blut und disseminierte Tumorzellen im Knochenmark sowie zirkulierende DNA und microRNAs, vor und nach Therapie, als potentielle Biomarker evaluiert. Durch Etablierung einer Vierfach-Immunfluoreszenzfärbung zum Nachweis LIN-28_{pos} und/oder SOX-2_{pos} Tumorzellen mit Stammzellcharakter im Knochenmark konnte die Präsenz dieser Zellen, sowohl vor als auch nach Chemotherapie, nachgewiesen werden. Dabei wurden sowohl epitheliale, als auch Tumorstammzellen ohne epithelialen Charakter detektiert, was die Vermutung nahe legte, dass sich einige dieser Zellen in Epithelialer-Mesenchymaler-Transition befanden, während epitheliale Tumorzellen mit Stammzellcharakter keine Umwandlung durchliefen oder den mesenchymalen Charakter wieder verloren hatten. Der mesenchymale Phänotyp konnte auch durch Untersuchungen an zirkulierenden Tumorzellen belegt werden, deren Prozentsatz dominierte und unter Therapie noch um 20% zunahm. Interessanterweise entwickelte sich unter Therapie ein PI3K/$Twist_{pos}$ Phänotyp, der vor Therapie nicht identifiziert wurde. Neben dem mesenchymalen- und Stammzellcharakter der zirkulierenden Tumorzellen konnte aber auch die Präsenz und Persistenz resistenter, $ERCC1_{pos}$ zirkulierender Tumorzellen belegt werden, wobei die Präsenz dieser Zellen mit einem verkürzten progressionsfreien Überleben, Gesamtüberleben und der klinischen Platinresistenz korrelierten. Durch Hinzunahme von ERCC1 als weiterem Marker auf zirkulierenden Tumorzellen konnte die Detektionsrate um etwa 15% erhöht werden. Somit scheinen Tumorzellen mit Stammzellcharakter, in Epithelialer-Mesenchymaler-Transition und resistente Populationen im Hinblick auf die schlechte Prognose des Ovarialkarzinoms bedeutend zu sein. Zusätzliche therapeutische Strategien könnten Signalwege der Tumorstammzellen angreifen, wie z.B. den PI3K/AKT Signalweg. Zusätzlich zu Studien über zirkulierende und disseminierte Tumorzellen werden die

Analysen von zirkulierender Tumor-DNA sowie microRNAs als ein „einfacheres“ Werkzeug diskutiert, um minimale Residualkrankheiten zu detektieren und die Erkrankung unter der gegebenen Therapie zu überwachen.

In dieser Arbeit wurde ein empfindliches und reproduzierbares Verfahren für die Next-Generation-Sequenzierung-basierte Analyse der zirkulierenden microRNA im Plasma von 15 platinsensiblen und 15 platinresistenten Patientinnen etabliert. Dabei wurden 102 potentiell neue miRNAs detektiert, von denen 22 mindestens in der Hälfte der Proben exprimierten. Die zehn am stärksten microRNAs, die sowohl in platinsensiblen und platinresistenten Proben exprimierten, waren: hsa-miR-128-3p, hsa-miR-99a-5p, hsa-let-7i-5p, hsa-miR-148a-3p, hsa-miR-129-5p, hsa-mir-381-3p, hsa-miR-9-3p, hsa-miR-9-5p, hsa-miR-433-3p, hsa-let-7b-5p. Bisher wurden im Vergleich der microRNA-Signatur von platinsensiblen und platinresistenten Patientinnen keine statistischen Unterschiede festgestellt.

Im Nebenprojekt der Promotion wurde der Methylierungsstatus von RASSF-1A an Tumorgeweben, im angrenzenden tumorfreien Gewebe und in korrespondierenden Plasmaproben mittels Real-Time Methylierungsspezifischer PCR und der semiquantitativen Methylation-Sensitive High-Resolution Melting Analysis, untersucht. In dieser Studie konnte gezeigt werden, das der RASSF-1A-Promoter im Plasma sowie im Tumor, aber auch im benachbarten tumorfreien Gewebe methyliert vorliegt, hier jedoch zu einem geringeren Prozentsatz. Die RASSF-1A-Promoter-Methylierung ergab prognostische Informationen in Bezug auf das Gesamtüberleben.

Abschließend scheint die Verwendung der sogenannten "Liquid Biopsy", hier Tumorzellen sowie zirkulierende DNA und miRNAs, für die Einschätzung der Prognose und die Überwachung vom Therapieverlauf des Ovarialkarzinoms attraktiv zu sein. In dieser Hinsicht können die Ergebnisse, die für die Zirkulation von DNA und microRNA erhalten wurden, die Ergebnisse für Tumorzellen ergänzen. Allerdings müssen diese Ergebnisse in klinischen Studien validiert werden, bevor sie für personalisierte Behandlungsstrategien verwendet werden können.

Summary

Despite advanced treatment options, the recurrence rate in ovarian carcinoma is approximately 75%. One of the major problems is the resistance to platinum-based chemotherapy in about 15-20% of cases which can only be recognized retrospectively. Thus, a very bad prognosis is attributed to ovarian cancer and it is, therefore, of great interest to develop innovative, blood-based biomarker concepts, which can provide information about prognosis and response during the course of therapy.

In this thesis, circulating tumor cells in blood samples and disseminated tumor cells in bone marrow as well as circulating DNA and microRNAs were evaluated as potential biomarkers in primary ovarian cancer patients before surgery and after platinum-based chemotherapy. Establishing a fourfold immunofluorescence staining method for the detection of LIN-28_{pos} and/or SOX-2_{pos} tumor cells with stem cell character in the bone marrow, the presence of these cells could be demonstrated before and after chemotherapy. Both, epithelial and non-epithelial tumor stem cells were detected suggesting that some of these cells were in Epithelial-Mesenchymal-Transition, while epithelial tumor cells with stem cell characteristics did not undergo any transformation or lost their mesenchymal character. The mesenchymal phenotype was also confirmed by studies on circulating tumor cells, representing the most frequently found phenotype which increased up to 20% under therapy. Interestingly, a PI3K/$Twist_{pos}$ phenotype developed under therapy that was not identified before therapy. In addition to mesenchymal and stem cell characteristics of the circulating tumor cells, the presence and persistence of more resistant, $ERCC1_{pos}$ circulating tumor cells could also be demonstrated. The presence of these cells correlated with a shortened progression-free survival, overall survival and clinical platinum resistance. By including ERCC1 as an additional marker on circulating tumor cells, the detection rate could be increased up to 15% before and after therapy. Thus, tumor cells with stem cell characteristics in Epithelial-Mesenchymal-Transition and resistant populations seem to play a role with regard to poor outcome of our ovarian cancer patients. Additional therapeutic strategies could target signalling pathways of the tumor stem cells, e.g. the PI3K/AKT signalling pathway. In addition to studies on circulating and disseminated tumor cells, the analyses of circulating tumor DNA as well as microRNAs is discussed to be a more convenient tool to detect minimal residual disease and monitor disease under the given therapy.

In this thesis, a sensitive and reproducible method for the Next-Generation-

Sequencing-based analysis of circulating microRNA in human plasma comparing 15 platinum-sensitive and 15 platinum-resistant cases was established. In this case, 102 potentially new miRNAs were detected, of which at least 22 expressed in the half of the samples. The ten most microRNAs expressing both platinum-sensitive and platinum-resistant samples were: hsa-miR-128-3p, hsa-miR-99a-5p, hsa-let-7i-5p, hsa-miR-148a-3p, hsa-miR-129-5p, hsa-miR-381-3p, hsa-miR-9-3p, hsa-miR-9-5p, hsa-miR-433-3p, hsa-let-7b-5p. Up to now, comparing the microRNA signature of platinum-sensitive and platinum-resistant patients, no statistical differences were detected.

In a sub project of this thesis, the methylation status of RASSF-1A in tumors, neighbouring tumor-free tissue and in corresponding plasma samples using real-time methylation-specific PCR and the semi-quantitative methylation-sensitive high-resolution melting analysis were investigated. Briefly, RASSF-1A promoter was observed in ctDNA and found highly methylated in primary tumors, and at lower percentages in the adjacent morphologically tumor cell-free tissues. We reported for the first time that RASSF-1A promoter methylation provided significant prognostic information in high grade serous ovarian cancer patients.

In conclusion, using the so-called "liquid biopsy", including tumor cells as well as circulating DNA and miRNAs, seem to be attractive for estimating prognosis and monitoring ovarian cancer. In this regard, results obtained for circulating DNA and microRNAs might complement results obtained for tumor cells. However, these results have to be validated in clinical trials before using them for personalized treatment strategies.

2 Einleitung

2.1 Das Ovarialkarzinom

Das Ovarialkarzinom ist die fünfthäufigste Todesursache bei Frauen mit einer Krebserkrankung in Europa und den Vereinigten Staaten (Goodman et al., 2003). Die Abb.2.1 zeigt eine Übersicht der relevanten Tumorerkrankungen in Deutschland, mit jährlich ca. 9000 Frauen, die mit einem Durchschnittsalter von 65 Jahren an einem Ovarialkarzinom erkranken, wobei ungefähr 6000 Frauen pro Jahr an den Folgen dieser Erkrankung sterben.

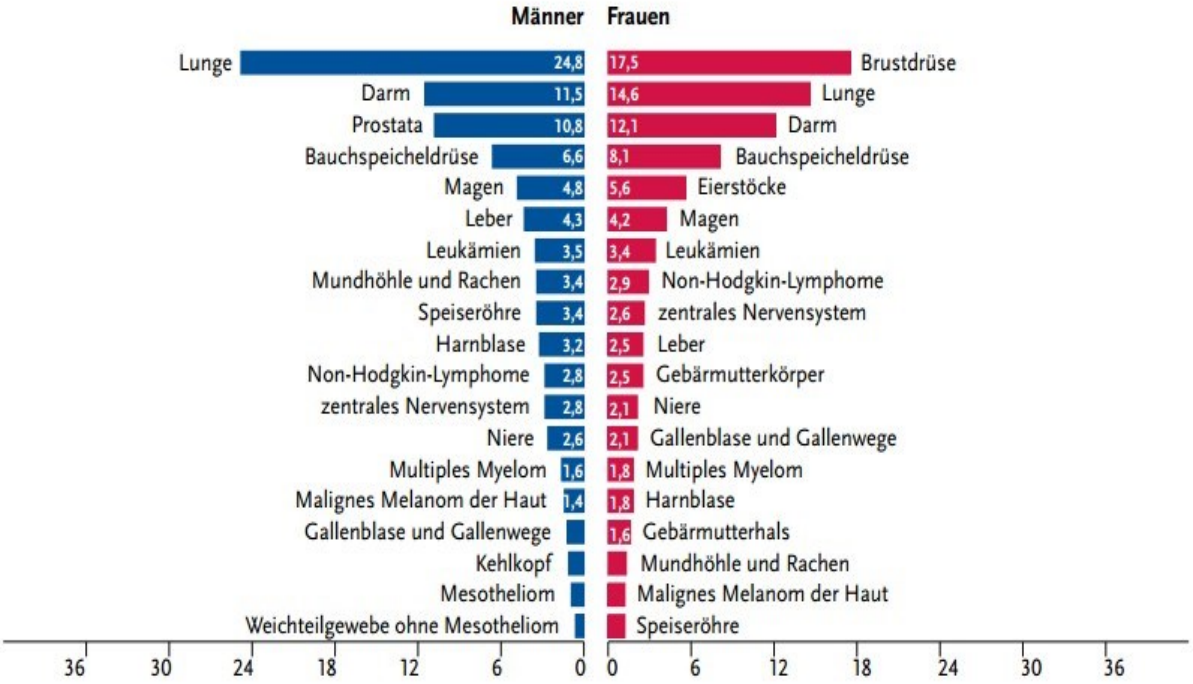

Abb.2.1 Prozentualer Anteil der häufigsten Tumorlokalisationen an allen Krebssterbefällen in Deutschland 2012 (Quelle: Robert Koch Institut)

Der Tumor wird gemäß der Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) in vier Klassen eingeteilt (Abb.2.2).

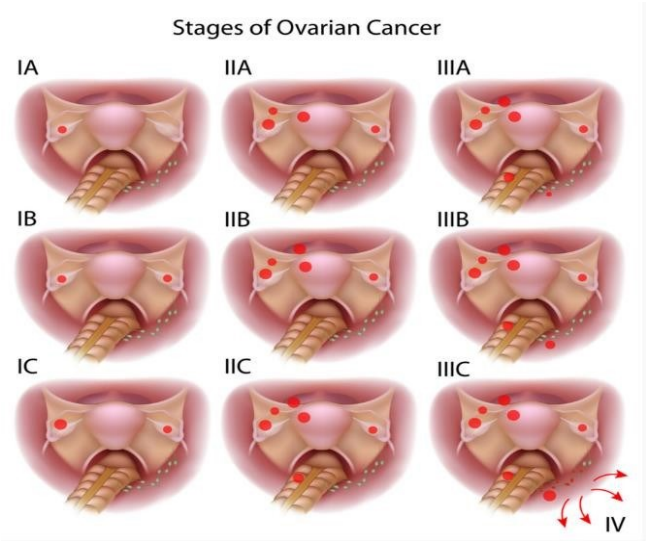

Abb.2.2 Übersicht der FIGO-Klassen (Quelle: http://nethealthbook.com/cancer-overview/ovarian-cancer/staging-ovarian-cancer/)

Tab.2.1 Übersicht der FIGO-Klassen (Prat et al., 2015)

FIGO I	Tumor befällt ein oder beide Eierstöcke
FIGO II	Tumor breitet sich in das Becken aus
FIGO III	Tumor breitet sich in der Bauchhöhle aus (peritoneale Metastasierung) oder befällt die Lymphknoten
FIGO IV	Tumor breitet sich über die Bauchhöhle hinaus aus (Fernmetastasierung)

Beschränkt sich der Tumor nur auf die Ovarien, so wird er dem Stadium I zugeordnet. Weitet sich die Tumorerkrankung auf das Becken aus, handelt es sich um eine Erkrankung des Stadiums FIGO II. Wird zusätzlich das Peritoneum (außerhalb des Beckens) befallen, wird FIGO III diagnostiziert und bei Bildung von Metastasen, die über die Bauchhöhle hinaus streuen, liegt eine Erkrankung des Stadiums FIGO IV vor (Tab.2.1). Aufgrund fehlender klinischer Frühsymptome und unzureichender Screening-Methoden, erfolgt die Erstdiagnose bei 75-80% der Fälle im fortgeschrittenen Stadium. Die Fünfjahres-Überlebensrate im Stadium III liegt bei 25-40% und im Stadium IV bei 11% (Forstner et al., 2016). Die meisten Ovarialkarzinome sind epithelialen Ursprungs (85-90%) und werden aufgrund der histomorphologischen Differenzierung in seröse, muzinöse, endometroide und klarzellige Karzinome

eingeteilt. Keimzelltumoren, Sarkome oder Stromatumoren spielen eine untergeordnete Rolle (Chan et al., 2006). Zu den Eckpfeilern des Therapiekonzeptes gehören die radikale Erstoperation mit dem Ziel der makroskopischen Komplettresektion (siehe Abb.2.3), sowie eine adjuvante Chemotherapie mit Carboplatin und Paclitaxel (Neijt et al., 1997; du Bois et al., 1999; 2005).

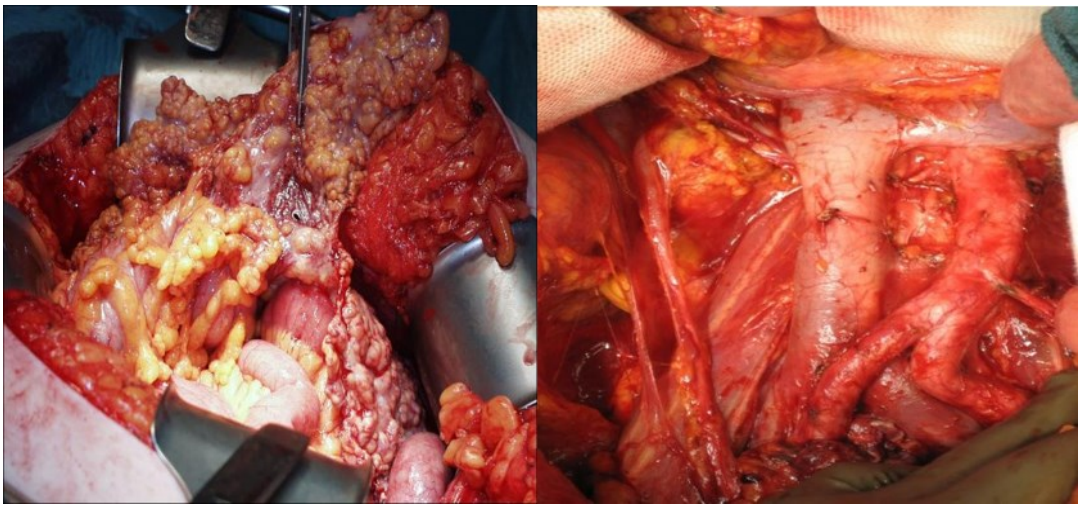

Abb.2.3 Vergleich vor (links) und nach (rechts) Operation

Die Standardtherapie des fortgeschrittenen Ovarialkarzinoms beinhaltet sechs Zyklen Carboplatin (AUC 5) und Paclitaxel (175 mg/m^2 KOF), die alle drei Wochen verabreicht werden. Der postoperative Tumorrest ist der stärkste unabhängige prognostische Faktor (Bristow et al., 2002; Wimberger et al., 2007, 2010). Sowohl die gesteigerte Radikalität des Primäreingriffs, als auch eine Platin- und Paclitaxelhaltige Kombinationstherapie haben dazu beigetragen, die progressionsfreie Zeit (PFS) sowie das Gesamtüberleben (OS) bei einem Teil der Patientinnen mit fortgeschrittenem Ovarialkarzinom zu verlängern (du Bois et al., 2005). Mittlerweile konnten neben diesen Standardtherapien multimodale Ansätze im Sinne einer individualisierten, zielgerichteten Therapie entwickelt werden. Neben Antikörpertherapien (Tab.2.2) kommen als zielgerichtete Therapien beim Ovarialkarzinom die „small molecules“ wie Sunitinib oder Sorafenib in Frage, die verschiedene Rezeptor-Tyrosinkinasen hemmen, die mit dem Tumorwachstum, der Angiogenese und der Entwicklung von Metastasen assoziiert werden. Zu den beteiligten Rezeptoren gehören der vaskuläre epitheliale Wachstumsfaktor (VEGF), der Stammzellwachstumsfaktor (C-Kit), der Plättchen-abgeleitete Wachstumsfaktor (PDGF), der fms-ähnliche Tyrosinkinaserezeptor (FLT), der Koloniestimulierende Faktor (CSF) und der neutropische Faktor (RET) (Kleibeuker et al., 2015; Prieto-Dominguez et al., 2016).

Tab.2.2 Antikörpertherapien für das Ovarialkarzinom

Antikörper	Zielantigen	Spezifität	Referenz
Bevacizumab	VEGF	Monospezifisch	Burger et al., 2007
Oregomovab	CA-125	Monospezifisch	Noujaim et al., 2001 Ehlen et al., 2005 Berek et al., 2009
Abagovomab	CA-125 Surrogat-Antigen	Monospezifisch	Pfisterer et al., 2006
HEA 125	EpCAMxCD3	Bispezfisch	Marme et al., 2002

Bezüglich der Antikörpertherapien sind monospezifische Antikörper am weitesten verbreitet. Hierzu zählen Bevacizumab (Avastin®) als ein rekombinanter, humanisierter Antikörper gegen VEGF (Burger et al., 2007), der seit 2011 für die Ersttherapie des Ovarialkarzinoms der Stadien FIGO III bis IV in Kombination mit Carboplatin und Paclitaxel zugelassen ist. Oregomovab (OvaREX, Mab-B43.13), ein muriner monoklonaler Antikörper, ist für das Tumorantigen CA-125 spezifisch und führt zu einer Formation zirkulierender Immunkomplexe (Noujaim et al., 2011; Gordon et al., 2013; Ehlen et al., 2005 und Berek et al., 2009). Abagovomab (ACA-125) ist ein antiidiotypischer, monoklonaler Antikörper, ebenfalls mit anti-CA-125 Spezifität (Pfisterer et al., 2006). Als bispezifische Antikörper sind HEA 125 (EpCAM) x OCT3 (CD3) (Staerz et al., 1986; Marme et al., 2002) und Catumaxomab, ein trifunktionaler, bisepzifischer Antikörper, (anti-EpCAM x anti CD3) zu nennen (Burges et al., 2007; Parsons et al., 2008; Wimberger et al., 2009). Der Wirkmechanismus des trifunktionalen Antikörpers erfolgt über die Bindung von Tumorzellen, die den epithelialen Oberflächenmarker epithelialer Zelladhäsionsmoleküle, EpCAM, als Antigen erkennen. Über die beiden weiteren Arme werden spezifisch T-Zellen (CD3-Antigen) und unspezifisch, über den FC-Teil, akzessorische Zellen, z.B. Makrophagen oder natürliche Killerzellen, gebunden (Abb.2.4). Diese Konstellation aktiviert verschiedene immunologische Wirkmechanismen, die zu einer komplexen Reaktion gegen die Tumorzellen führen.

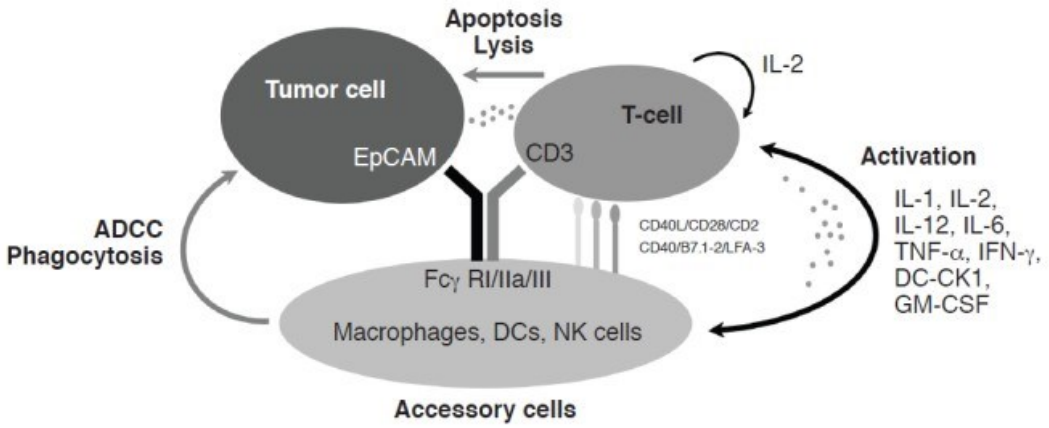

Abb.2.4 Übersicht der Wirkungsweise von Catumaxumab (Seimetz et al., 2011)

Trotz fortgeschrittener Behandlungsmöglichkeiten beträgt die Rezidivrate beim Ovarialkarzinom etwa 75%. Das größte Problem stellt die Platinresistenz dar, die bei 15-20% der Patientinnen auftritt. Bisher lässt sich die Platinresistenz nur retrospektiv beschreiben (Bookmann et al., 1999). Grundsätzlich werden zwei Gruppen von Patientinnen bezüglich des Rezidivs unterschieden. Die Gruppe der Platinresistenten beschreibt die Patientinnen, die innerhalb von sechs Monaten nach Abschluss der Chemotherapie ein Rezidiv entwickeln. Häufig werden diese Patientinnen mit einer Monotherapie, wie z.B. Paclitaxel oder Gemcitabine, weiter behandelt. Anders stellt sich die Situation der platinsensiblen Patientinnen dar, die ein Rezidiv nach mehr als sechs Monaten nach Abschluss der Chemotherapie erleiden. Hier ist es üblich, den Patientinnen eine platinhaltige Kombinationstherapie, wie Carboplatin/Gemcitabine, anzubieten. Vor kurzem wurde der Poly (ADP-RibosePolymerase1)-Inhibitor (PARP1-Inhibitor) Olaparib von der Food and Drug Administration (FDA) in den USA und der European Medicines Agency (EMA) zur Therapie des Ovarialkarzinoms zugelassen (Meehan et al., 2016). Diese Therapie wird bei Patientinnen mit Rezidiv eines fortgeschrittenen Ovarialkarzinoms, die nachweislich eine krankheitsverursachende Mutation in BRCA1- oder BRCA2-Genen aufweisen, durchgeführt.

Insgesamt hat das Ovarialkarzinom eine sehr schlechte Prognose und es ist daher umso wichtiger, neue Biomarkerkonzepte zu entwickeln, die Aufschluss über Prognose, Rezidiventwicklung bzw. zielgerichtete Therapien geben könnten. Die Ursachen für die schlechte Prognose beim Ovarialkarzinom sind multifaktoriell. Ein Faktor ist die hämatogene Tumorzellstreuung, repräsentiert durch disseminierte Tumorzellen (DTZ) im Knochenmark (KM) und zirkulierende Tumorzellen im Blut

(ZTZ). Obwohl die prognostische Bedeutung dieser Zellen bekannt ist, gibt es beim Ovarialkarzinom kaum Daten zur Charakterisierung dieser Zellen.

2.2 Tumorzelldifferenzierung

Die Tumorzelldisseminierung beschreibt im Allgemeinen die Streuung der einzelnen Tumorzellen aus dem Primärtumor, welche über das Blut- und Lymphgefäßsystem organisiert ist. Einen schematischen Überblick zeigt die Abb.2.5. Dabei verlassen Tumorzellen in frühen Stadien den Primärtumor und gelangen über die Blutbahn in sekundäre Organe, wie z.B. das KM. Dort verweilen sie in Form einzelner DTZ oder in Form von Zellaggregaten, den Mikrometastasen. Mit den DTZ wird sehr häufig der Begriff *„Dormancy“* in Verbindung gebracht. Es wird zwischen *Tumor-Cell-Dormancy* und *Tumor-Mass-Dormancy* unterschieden. Ersteres beschreibt die Fähigkeit der Tumorzellen, sich in einem ruhenden, nicht proliferativen Zustand zu organisieren. Die *Tumor-Mass Dormancy* hingegen beschreibt ein Gleichgewichtszustand zwischen Proliferation und Apoptose der Mikrometastasen. Die ruhenden DTZ können wieder aktiviert werden, in die Blutzirkulation eintreten und eine Metastase bilden. Wann dies geschieht, und unter welchen Bedingungen, ist weitgehend unbekannt. In der Theorie gibt es bisher drei Modelle, die die Entstehung einer *Dormancy* erklären (Bragado et al., 2012):

1. Stressfaktoren im Tumor, z.B. Hypoxie, wirken auf primäre Tumorzellen und programmieren neuerlich disseminierende Tumorzellen schon im Vorfeld auf den Zustand der *Dormancy*.
2. Eine frühe Tumorzelldisseminierung bedingt eine DTZ-Population, die noch unfähig für ein metastatisches Wachstum ist, somit in einem „Arrestzustand“ verharrt und erst weitere genetische Veränderungen für eine klonale Expansion erwerben muss.
3. DTZ invasiver Karzinome antworten mit Stresssignalen auf die Disseminierung bzw. auf einen wachstumsinhibierenden Einfluss des extrazellulären Milieus im Zielorgan und induzieren *Dormancy*.

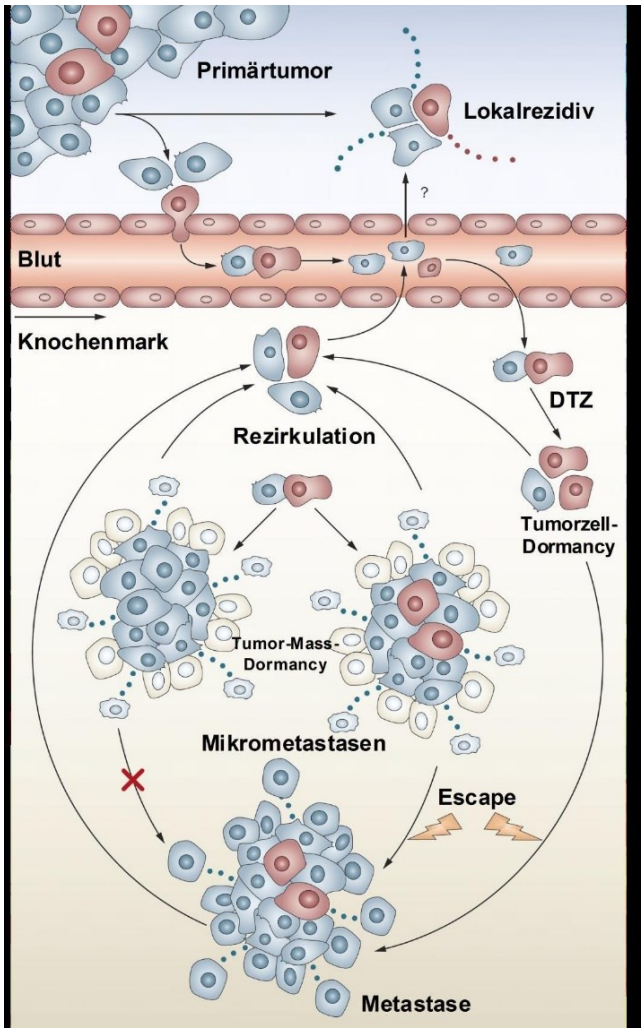

Abb.2.5 Konzept der Tumorzellmetastasierung nach Pantel et al., 2009

Es ist noch nicht genau verstanden, welche Faktoren dazu beitragen, eine sich in *Dormancy* befindliche DTZ wieder in einen proliferativen Zustand zu versetzen. So wird auch vermutet, dass in erster Linie DTZ mit Stammzelleigenschaften in der Lage sind, eine Metastase bzw. ein Rezidiv zu bilden (Sosa et al., 2014). Beim Mammakarzinom konnte die Präsenz von DTZ mit Stammzellcharakter bereits gezeigt werden (Balic et al., 2006, Reuben et al., 2011). Beim Ovarialkarzinom gibt es bisher keine Daten zu DTZ mit Stammzelleigenschaften. Weiterhin beherrschen Tumorzellen die Fähigkeit zu einer Epithelialen-Mesenchymalen-Transition (EMT), bei dem eine Tumorzelle ihren epithelialen Charakter verliert und einen mesenchymalen Phänotyp annimmt (Abb.2.6). Somit kann diese Zelle „unerkannt“ zirkulieren und später, nach Einwanderung in sekundäre Organe, wieder zu einer epithelialen Zelle konvertieren (Romero-Laorden et al., 2014).

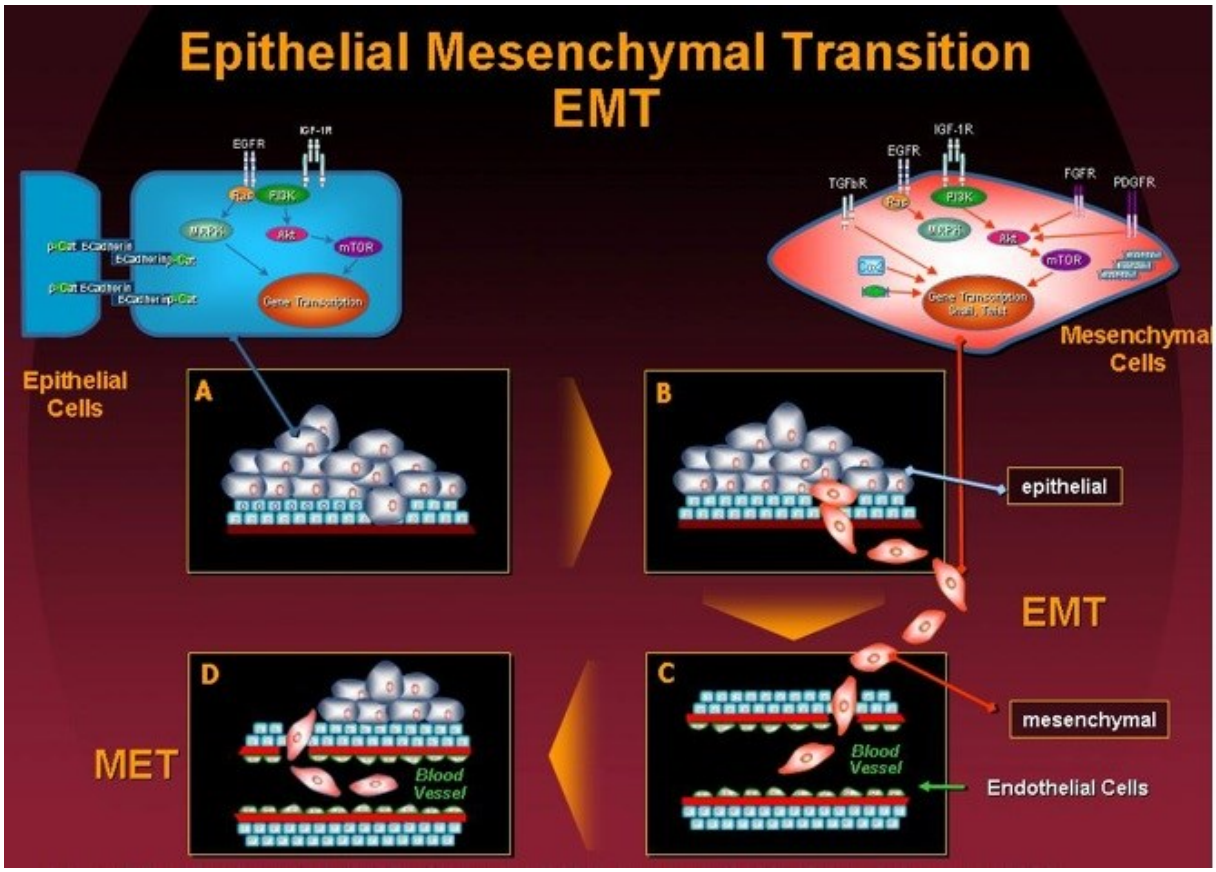

Abb.2.6 Schematische Darstellung der Epithelialen-Mesenchymalen-Transition (Brabletz et al., 2005; Christofori et al., 2006; Thiery & Sleemann 2006)

2.3 Disseminierte Tumorzellen im Knochenmark und zirkulierende Tumorzellen im Blut beim Ovarialkarzinom

Die Präsenz von DTZ ist ein häufiges Phänomen in soliden Tumoren epithelialen Ursprungs und ist gerade beim Mammakarzinom mit einer schlechten Prognose assoziiert (Braun et al., 2005; Janni et al., 2011; Hartkopf et al., 2014). Auch beim Ovarialkarzinom gibt es Hinweise, dass die hämatogene Disseminierung ein ungünstiger Prognosefaktor ist (Tab.2.3).

Tab.2.3 Übersicht der Studien zum Thema disseminierte Tumorzellen

Autor	Anzahl der Patienten	Methode	Median follow-up [months]	Pos. Rate (%)	Prognostische Signifikanz
Marth et al., 2002	73	DTZ (Immunobeads)	25	21	Keine Signifikanz
Braun et al., 2001	108	DTZ (ICC)	45	30%	DFS
Schindlbeck et al., 2007	90	DTZ (ICC)	28	23%	DDFS
***Wimberger et al., 2007**	62	DTZ (ICC)	18	54%	DFS
Banys et al., 2009	112	DTZ (ICC)	12	25%	DFS
***Fehm et al., 2013**	495	DTZ (ICC)	46	27%	PFS,OS

***: Publikationen unter Beteiligung der Frauenklinik Essen**

Neben der Frauenklinik Essen konnten auch andere Gruppen zeigen, dass die Detektionsrate von DTZ im Ovarialkarzinom, bei identischer Methodenwahl, zwischen 20 bis 55% liegt und mit einer ungünstigen Prognose assoziiert ist (Braun et al., 2001; Schindlbeck et al., 2007; Wimberger et al., 2007; Banys et al., 2009 und Fehm et al., 2013). Außerdem konnte die Frauenklinik Essen zeigen, dass DTZ nach Therapie EpCAM-positiv, und nicht apoptopisch sind (Wimberger et al., 2007). Jedoch ist die Methode sehr invasiv und für ein Therapiemonitoring eher ungeeignet.

Deshalb wird verstärkt versucht, Biomarker im Blut zu identifizieren. Hierzu zählen ZTZ, für die im Gegensatz zur Detektion von DTZ noch keine Standardmethode klassifiziert wurde. Mittlerweile gibt es mehr als 40 Methoden, um ZTZ im Blut zu selektieren bzw. anzureichern (Alix-Panabieres & Pantel, 2014). Deshalb werden für die Identifikation und Charakterisierung sehr sensitive und spezifische Analysemethoden benötigt. Die am häufigsten verwendeten Selektionsmethoden sind in Abb.2.7 dargestellt.

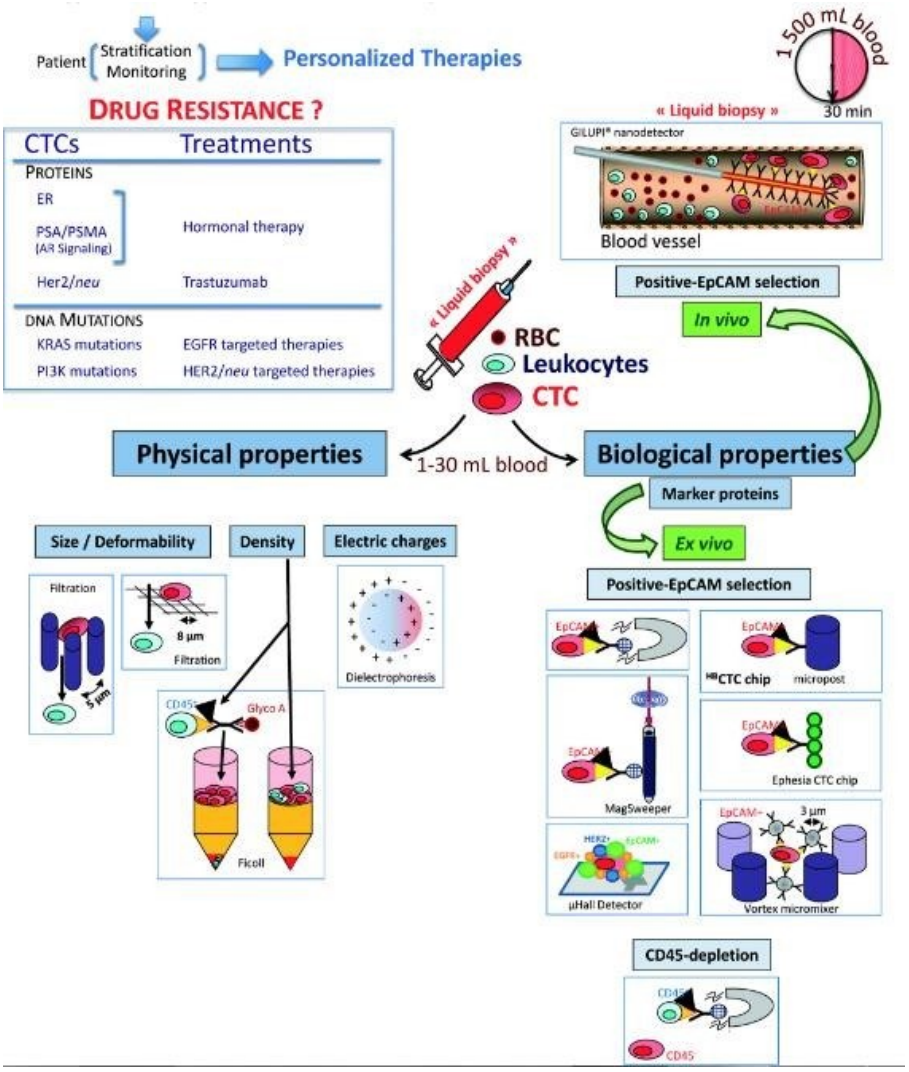

Abb.2.7 Selektionstechniken zur ZTZ Gewinnung

ZTZ werden durch eine Flüssigkeitsbiopsie aus dem Blut entnommen und mit verschiedenen Methoden angereichert. A) Physikalische Eigenschaften beinhalten die Größe, Deformation, Dichte und elektrische Ladung. B) Biologische Eigenschaften basieren auf der Expression von Zelloberflächen-markern z.B. EpCAM zur positiven Selektion und CD45 zur negativen Selektion. Legende: glykoA (Glycophorin A), RBC (rote Blutzellen), PSA (Prostata-spezifisches Antigen), PSMA (Prostataspezifisches Memebranantigen, AR (Androgenrezeptor), EGFR (Endothelialer Wachstumsfaktor) (Alix-Panabieres & Pantel, 2014).

Die Tumorzellanreicherung berücksichtigt unterschiedliche Parameter, wie z.B. physikalische Eigenschaften (Größe, Dichte, elektrische Ladung, Deformabilität) sowie biologische Eigenschaften (Expression der Oberflächenproteine). Zu den physikalischen Methoden gehören die Dichtegradientenzentrifugation, spezielle Filter, die nach Zellgrößen selektieren wie z.B. „ISET“ (Isolation by size of epithelial tumor cells) sowie die dreidimensionalen Mikrofilter. Neue Methoden basieren auf sogenannten „Biochips“, die den Unterschied der Größe und Deformation von ZTZ, im Gegensatz zu den Blutzellen, nutzt und mikrofluidale Systeme, wie „Multi-Orifice

Flow Fractionation“ (MOFF), „Dielectrophoretisis“ (DEP) und „Dielectrophoretisis Field-Flow Fractionation“ (Dep-FFF), die mit Hilfe der Dielektrophorese die unterschiedlichen Größen und Membraneigenschaften der ZTZ erkennen. Biologische Anreicherungsmethoden basieren auf der Selektion der Tumorzellen anhand von Antikörpern, die an Magnetbeads gekoppelt sind und tumorassoziierte Antigene auf ZTZ binden (positive Selektion) oder die Leukozyten (CD45) aus dem Blut abreichern (negative Selektion). Weitere, auf „ZTZ-Chips“ basierende, Methoden gewinnen $EpCAM_{pos}$ ZTZ über eine Kombination aus größen-basierender, hydrodynamischer Zellsortierung und der immunmagnetischen Selektion (positiv/negativ). Eine *in vivo* Methode ist der „GILUPI® Nanodetector“, der es mit einer Antikörper-beschichteten Braunüle in der Armvene für 30 Minuten ermöglicht, mit 1500 ml Blut eine Anreicherung der ZTZ durchzuführen. Neben den Vorteilen beherbergen diese Systeme auch Nachteile, die aufgrund der Heterogenität der Tumorzellen oftmals eine schlechte Wiederfindungsrate mit sich bringen. Des Weiteren beeinflussen Luftblasen und der Verlust an Oberflächenantigenen die Selektion mittels Magnetbeads. Eine Verklumpung der Zellen erschwert die Selektion bei den „ZTZ-Chips“ und den Filtern.

Die nachfolgende Abbildung (Abb.2.8) zeigt einen schematischen Überblick über die Gruppe der bildbasierenden- und molekularbiologischen Detektionsmöglich-keiten. Die zurzeit einzige, von der FDA 2004, zugelassene Methode ist das CellSearch® System, bei der ZTZ aus 7,5 ml Blut anhand von EpCAM beschichteten, magnetischen Beads isoliert werden. Der Nachweis erfolgt mittels Immunfluoreszenz der ZTZ mit einem pan-Zytokeratin-Antikörper (epithelialer Marker), DAPI (Marker für den Zellkern) und einem anti-CD45-Antikörper, um die unspezifische Detektion falsch-positiver Leukozyten auszuschließen. Das AriolSystem, ein automasiertes Bildanalysesystem, dient der Quantifizierung von ZTZ auf Objektträgern. Mittels der Laser-Scan-Mikroskopie werden Präparate von einem fokussierten Laserstrahl abgetastet, wodurch die Fluoreszenzemission der Probe detektiert wird. Eine weitere Option zur bildbasierenden Methode stellt die EPISPOT-Methode dar. Sie detektiert tumorsepezifische Proteine, die von ZTZ freigesetzt werden. Die Detektion mittels molekularer Analysesysteme basieren auf der RNA-Analyse lebender Tumorzellen und anschließender Reverser Transkriptase-Polymerase-Kettenreaktion (RT-PCR) sowie der Amplifikation von tumor- und epithelspezifischen Markern. Durch die Flexibiltät der Multiplex-

Untersuchungen lässt sich eine größere Anzahl von tumorassoziierten Markern gleichzeitig bestimmen (Lianidou & Markou et al., 2011). Auch die Detektionssysteme besitzen Vor- und Nachteile. So werden mit dem CellSearch® System zwar ZTZ quantifiziert, jedoch nur $EpCAM_{pos}$ ZTZ angereichert. Eine Detektionsalternative stellt die PCR dar, jedoch erlaubt sie keine akkurate Quantifizierung der ZTZ, da eine unterschiedliche Anzahl von Transkripten ebenfalls von Nicht-Tumorzellen exprimiert werden kann (Lianidou & Markou et al., 2011). Hier ist die Etablierung sensitiver Methoden essentiell, um auch den Leukozytenhintergrund zu reduzieren, der falsch-positive Ergebnisse generieren kann. Ein genereller Vorteil ist hier jedoch die Möglichkeit, durch Multimarkeranalysen die Heterogenität der ZTZ zu erfassen.

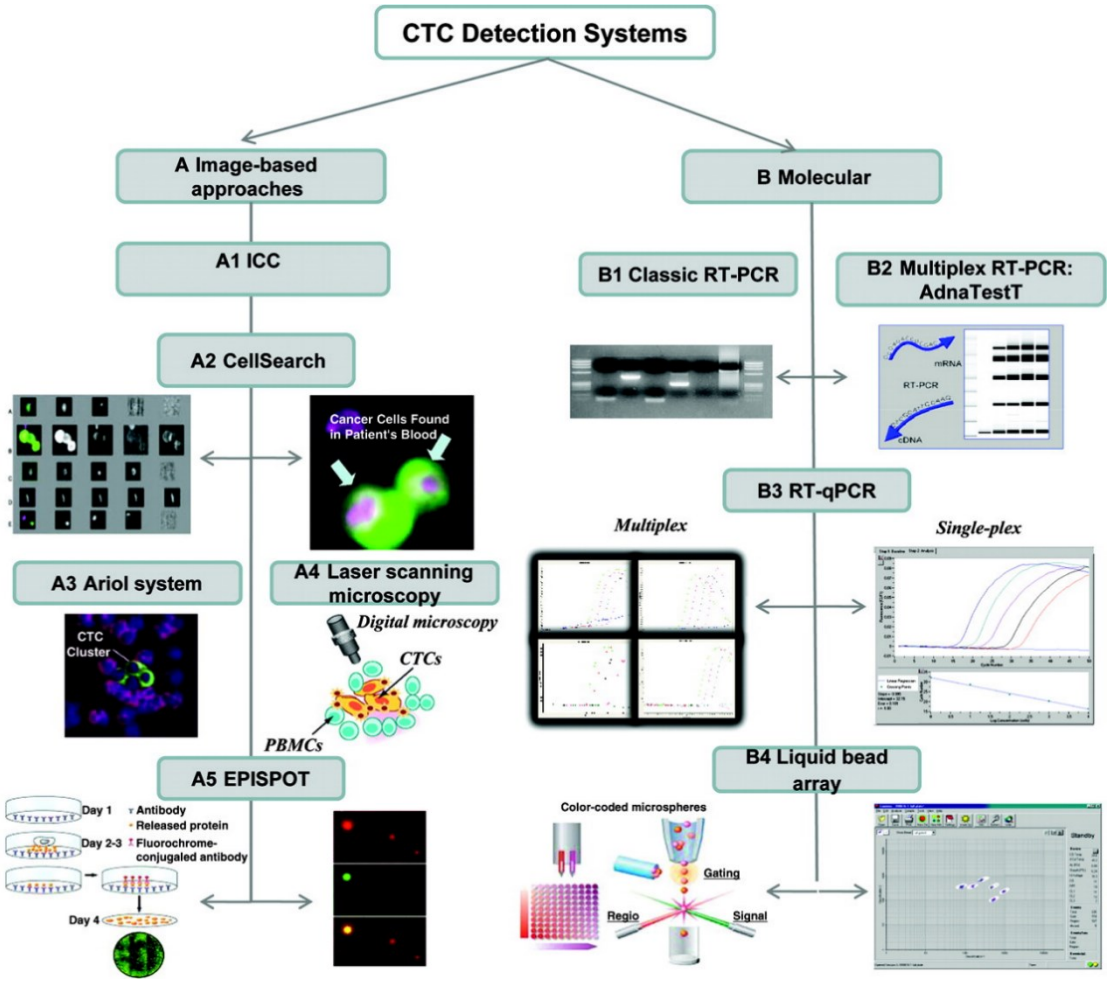

Abb.2.8 Detektionssysteme für ZTZ

A) bildbasierende Methoden: Immunzytochemie (ICC), CellSearch® System, AriolSystem, Laser-Scanning-Mikroskopie, EPISPOT, B) Molekulare Methoden: Singleplex-RT-PCR, Multiplex RT-PCR (AdnaTest) sowie eine RT-qPCR und die Flüssigkeit-Bead-Arrays **(Lianidou & Markou, 2011).**

Beim Ovarialkarzinom gibt es nur wenige Studien, die sich mit der Selektion und Detektion der ZTZ beschäftigt haben. Die Tab.2.4 zeigt die bisher publizierten Studien, bei der die Methodenwahl sehr heterogen war und die Positivitätsrate somit zwischen 20 bis 90% lag.

Tab.2.4 Übersicht der Studien zum Thema zirkulierende Tumorzellen

Autor	Methode	Antikörper/ Marker	Anzahl der Patienten	Pos. Rate (%)	Prognostische Signifikanz
Kurata et al., 2002	RT-PCR	CK7,CK20	24	46	No
Marth et al., 2002	Dichtegradient Immunmagnetische Beads	MOC-31	90	12	No
Sapi et al., 2002	Immunmagnetische Bead	HEA-125	20	75	No
Judson et al., 2003	Dichtegradient Immunmagnetische Beads/ICC	CK7,8,18, 20 TFS-2;EGFR	64	19	No
Oikonomopoulou et al., 2006	Immunmagnetische Beads/RT-PCR	BER-EP4 Kalikreins 6,10	24	75	No
***Wimberger et al., 2007**	Dichtegradient/ ICC	A45-B/B3 CK8,18,19	57	21	No
He et al., 2008	Dichtegradient/ Flowzytometrie	Folate-Alexa-Flour 488 DUPA-FITC	20	90	No
Fan et al., 2009	Zellinvasionassay	-	71	61	Yes (PFS)
***Aktas et al., 2011**	RT-PCR	EpCAM, MUC1,HER2	86 Primärdia. 70 n.Chemo.	19 27	Yes (OS) Yes (OS)
Obermayr et al., 2012	Dichtegradient RT-qPCR	Multimarker Genpanel	216 Primärdia. Während Follow-up	24 20	No (PFS,OS) Yes (PFS,OS)
Liu et al., 2013	Cellsearch	CK 8,18,19	78	80	No
Kuhlmann et al., 2014	RT-PCR	EpCAM, MUC1,HER2	143	14	Yes (OS)

***:Publikation unter Beteiligung der Frauenklinik Essen**

Die prognostische Bedeutung im Hinblick auf das PFS und OS konnte nur in vier Studien gezeigt werden, wobei auch die Methoden unterschiedlich waren (Fan et al., 2009; Aktas et al., 2011; Obermayr et al., 2012, Kuhlmann et al., 2014). Im Gegensatz dazu haben alle weiteren, in der Tabelle zusammengefassten, Arbeitsgruppen keine prognostische Bedeutung der ZTZ zeigen können. Interessanterweise konnte die Frauenklinik Essen kürzlich publizieren, dass die Expression vom „Excision-Repair Cross-Complementation Group 1 (ERCC1) Protein, einem DNA-Reparaturenzym auf ZTZ, im Blut von Ovarialkarzinompatientinnen zu Beginn der Erkrankung als prädiktiver Marker für ein verkürztes PFS und OS sowie eine klinische Platinresistenz identifiziert wurde (Kuhlmann et al., 2014).

2.4 Resistenzmechanismen und prognostische Bedeutung von ERCC1 im Ovarialkarzinom

Insgesamt gibt es vier bekannte DNA-Reparaturmöglichkeiten, um eine Zelle vor Chromosomenaberrationen zu schützen. Neben der Einzelstrang- und Doppelstrang-reparatur verfügt eine Zelle noch über die Möglichkeiten der Photoreaktivierung und Reparatur von Quervernetzungen. Die Nukleotidexzisionsreparatur ist ein hoch konservierter Einzelstrang DNA-Reparatur-Signalweg, der vor allem „bulky lesions“ erkennt. Dies sind Lokalisationen, die eine Art „Buckel“ erzeugen und dadurch die Helixstruktur stören. Ursachen sind Pyrimidindimere und 6,4 Photoprodukte sowie Cisplatin, die durch UV-Strahlen verursacht werden. Die Reparatur gliedert sich in drei Abschnitte (Abb.2.9):

1. Schadenserkennung durch einen DNA-Bindungsfaktor und Herausschneiden einer 25-30 Basen langen DNA-Sequenz.
2. Neusynthese der Sequenz und anschließende Ligation. Das ERRC1 Enzym spielt eine wichtige Schlüsselrolle in der Reparatur. Es dimerisiert mit dem Molekül Xeroderma Pigmentosum Komplementation Gruppe F (XPF) und wird benötigt, um den schädlichen DNA-Strang zu exzidieren (Shi et al., 2012). Die Bindung ist für die Stabilität und die katalytische Aktivität von XPF wichtig, da ansonsten das ERCC1 nicht am 5´Ende und XPF am 3´-Ende die DNA restriktieren kann (Gossage & Madhusudan et al., 2007).

3. Die DNA-Polymerase synthetisiert den komplementären Strang, sodass die DNA-Helix ihre ursprüngliche Form zurückerhält.

Bisher gibt es nur wenige Studien, die die Expression und die prognostische Bedeutung von ERCC1 im Ovarialkarzinom untersucht haben. In einer Studie mit Tumorgeweben von 101 Ovarialkarzinompatientinnen wurde in 14% der Fälle eine Überexpression von ERCC1 detektiert, wobei 75% der Patientinnen eine Platinresistenz entwickelten, was mit einem signifikant reduzierten PFS und OS korrelierte (Steffensen et al., 2009). In einer ähnlichen Studie war die ERCC1-Expression im Tumorgewebe bei Patientinnen mit Platinresistenz signifikant höher als in der Gruppe der platinsensitiven Patientinnen, wobei keine prognostische Relevanz beobachtet wurde (Xie et al., 2011). Ovarialkarzinompatientinnen mit einer höheren ERCC1 Expressionsrate im Gewebe und adjuvanter, platinbasierter Chemotherapie hatten ein signifikant kürzeres PFS und OS als die Gruppe mit niedriger ERCC1-Expression (Milivic-Kovacevic et al., 2011). Eine Schlüsselpublikation im *New England Journal of Medicine* berichtete über eine umfassende Neubewertung von über 494 Patienten mit Lungenkarzinom und folgerte, dass die immunhistochemische ERCC1 Erkennung im Primärtumor mit allen derzeit verfügbaren Antikörpern für Kliniker in Bezug auf die Vorhersage von Platinresistenz und Therapieentscheidungen ungeeignet ist (Friboulet et al., 2013). Die Arbeitsgruppe der Frauenklinik Essen hatte auf Gewebeebene auch eine ERCC1 Expression beim Ovarialkarzinom zeigen können, jedoch wurde keine Korrelation im Hinblick auf das PFS, OS und die Platinresistenz gefunden. Im Gegensatz dazu zeigte sowohl die univariable, als auch die multivariable Analyse der Präsenz von $ERCC1_{pos}$ ZTZ im Blut eine signifikante Korrelation mit einem verkürzten PFS, OS und der Platinresistenz (Kuhlmann et al., 2014).

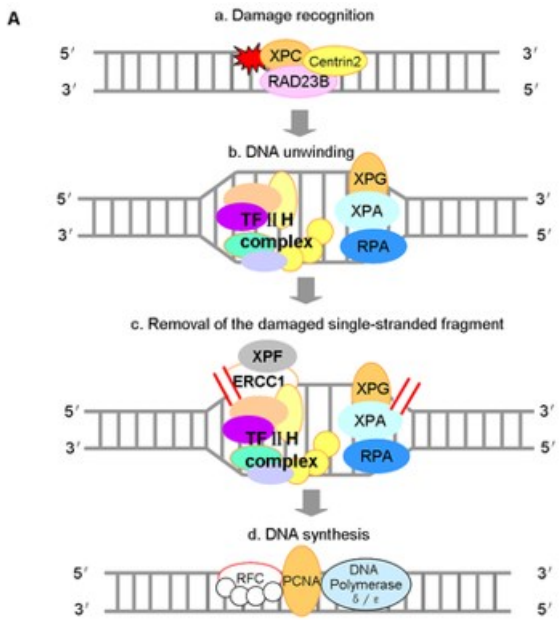

Abb.2.9 Übersicht der DNA-Reparatur durch den ERCC1-XPF Komplex (Shi et al., 2012)

Da die Gewinnung der DTZ für die Verlaufskontrolle einer Patientin zu invasiv sind und die Selektion und Charakterisierung der ZTZ sehr aufwendig ist und keiner Standardmethode unterliegt, wird im Rahmen der sogenannten „Liquid Biopsy“ auch die Analyse der zirkulierenden Tumor DNA (ztDNA) sowie der sogenannten microRNAs (miRNA) favorisiert. Deren genetische oder epigenetische Analysen in Form von Methylierungs-, Heterozygosität- und miRNA- Analysen könnten dazu beitragen, die Progression von Tumoren und die Bildung von Metastasen zu fördern (Qu et al., 2011; Kuhlmann et al., 2012; Marzese et al., 2013 und Warton et al., 2015).

2.5 Zirkulierende freie DNA

Erstmalig wurde im Jahre 1948 die Existenz von freier, zirkulierender DNA (zfDNA) beschrieben (Mandel und Métais et al., 1948). Allerdings geriet das Feld in Vergessenheit und wurde in den 1970er Jahren bei Patientinnen mit malignen Tumoren, die eine höhere DNA-Konzentration aufwiesen als Patientinnen ohne Tumorerkrankung, wiederentdeckt (Leon et al., 1977). Die ztDNA macht prozentual nur einen kleinen Teil der gesamten zfDNA im Blut aus und wird vom Primärtumor, Mikrometastasen oder von apoptopischen bzw. nekrotischen Zellen freigesetzt, nicht nur in freier Form, sondern auch in Form von Nukleosomen, die im peripheren Blut zirkulieren (Roth et al., 2011; Panabiéres et al., 2012) (Abb.2.10a).

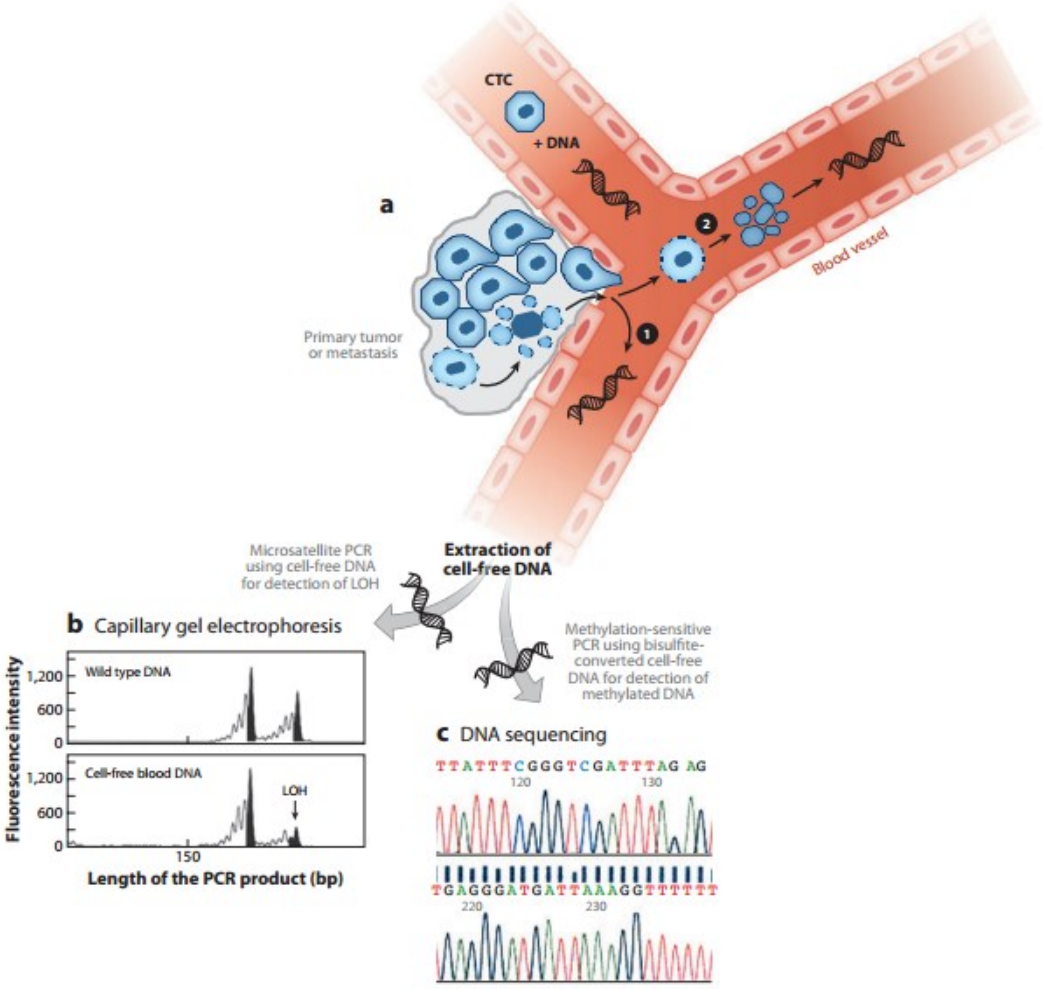

Abb.2.10 Detektion von genetisch und epigenetisch veränderter DNA im Blut. A: ZtDNA, die entweder vom Primärtumor, Mikrometastasen oder apoptopischen und nekrotischen ZTZ stammt, wird in das Blut freigesetzt.B. Kapillarelektrophorese als eine Möglichkeit zur Detektion des Verlustes der Heterozygosität bei zfDNA; C: Sodiumbisulfit Methode als ein Beispiel für die epigentische Analyse von zfDNA **(Panabiéres et al., 2012).**

Aus dem Serum bzw. Plasma lässt sich das genetische Material extrahieren und Charakteristika der DNA aus den Tumorzellen nachweisen. Dadurch können tumorspezifische Analysen in Form von Mutationsanalysen, Mikrosatelliten-stabilitätsanalysen, der Verlust der Heterozygosität und DNA-Methylierungsanalyse, durchgeführt werden (Abb.2.10 b,c). Aufgrund der geringen DNA-Ausgangsmenge sind sehr sensible Detektionsverfahren notwendig, die auf dem Prinzip der Digitalisierung der Messsignale basieren.

Neben der Real Time-PCR, ELISA und Fluoreszenzfärbungen stehen das Next-Generation-Sequencing (NGS), die branched DNA-Technik (bDNA) und die **BEAM**ing-PCR (**Beads**-**Emulsion**-PCR, **Amplifikation**, **Magnetic** Beads) zur Verfügung. Letztere basiert auf einer Kombination von digitaler PCR und Durchflusszytometrie

(Diehl et al., 2005; Shao et al., 2015; Panabieres et al., 2016). Eine weitere, häufig angewendete Methode, die für die epigenetische Analyse genutzt wird, ist die „Sodiumbisulfit“ Behandlung von extrahierter DNA, die unmethylierte Zytosine zu Uracil konvertiert und im Anschluss über die Methylierungsspezifische PCR (MSP) amplifiziert. Die Primerpaare werden als "methylierungsspezifisch" konzipiert, indem sie Sequenzen enthalten, die nur nicht umgesetzte 5-Methylzytosine, oder umgekehrt "nicht methylierte", komplementäre Thymine, die aus unmethylierten Zytosinen umgesetzt sind, ergänzen. Die Methylierung wird durch die Fähigkeit des spezifischen Primers zur Verstärkung bestimmt.

2.6 Zirkulierende freie DNA und das Ovarialkarzinom

Studien an zfDNA wurden in den letzten Jahren auch bei Patientinnen mit epithelialem Ovarialkarzinom durchgeführt, wobei in einigen Fällen die Ergebnisse diskrepant waren (Zhou et al., 2016). Die Diskrepanzen begründen sich durch unterschiedliche Methodiken und voranalytischen Bedingungen, die Verwendung von Serum anstelle von Plasma und die verschiedenen Volumina von Plasma/Serum für die zfDNA-Extraktion. Viele Studien konzentrierten sich auf die potenzielle Verwendung von zfDNA als diagnostischen, prognostischen und prädiktiven Biomarker beim Ovarialkarzinom, und die kürzlich publizierte Metaanalyse evaluierte das Potential der zfDNA als nicht invasiven Biomarker (Zhou et al., 2016). Eine Studie zur zfDNA im Rahmen der Vorsorge zielte darauf ab, die Plasma-zfDNA unter Verwendung eines Real-Time-PCR-Assays für drei Referenzgene zu quantifizieren. Die Daten zeigten, dass zfDNA in Plasmaproben von Patientinnen mit fortgeschrittenem Ovarialkarzinom, im Vergleich zu Kontrollen, erhöht vorlagen (Kamat et al., 2006). Ferner konnte mittels bDNA-Analyse im Plasma gezeigt werden, dass im Vergleich zu Patientinnen im frühen Stadium, die Serum-zfDNA-Konzentration bei Patientinnen im fortgeschrittenen Stadium signifikant höher war (Shao et al., 2015). Eine weitere Gruppe untersuchte den prognostischen Wert von zfDNA im Serum und zeigte keinen signifikanten Unterschied zwischen zfDNA-Konzentrationen von Patientinnen mit benignen und malignen Erkrankungen (No et al., 2012).

Neben der Charakterisierung der ztDNA durch den Verlust der Heterozygosität spielen auch epigenetische Veränderungen in Form von Methylierungen eine Rolle. Die epigenetische Inaktivierung eines Tumorsuppressorgens resultiert oft aus seiner Promotormethylierung und wird als ein frühes Ereignis während der Karzinogenese angesehen (Jones et al., 2002). Ein häufig in diesem Kontext untersuchtes Gen ist das

RASSF1-Gen (RAS-Assoziation Domain-haltiges Protein 1), das zur Ras-Assoziationsdomäne-Familie gehört, bestehend aus zehn Mitgliedern. RASSF-Proteine tragen zur Mikrotubulistabilität bei und sind in Zellzyklusregulation, Apoptose, Zellmigration und Zelladhäsion involviert. Das RASSF1-Gen befindet sich auf dem 3p21.3-Locus und umfasst acht Exons. Seine zwei Promotorregionen und das alternative Splicing sind für die acht Isoformen A-H verantwortlich. RASSF1A und RASSF1C sind bisher weitestgehend untersucht. Insbesondere die RASSF1A-Gen-Isoform, die als Tumorsuppressor bei humanen Malignomen identifiziert wurde (Richter et al., 2009; Volodko et al., 2014). RASSF1A beeinflusst zahlreiche Signalkaskaden, z.B. Ras/PI3K/AKT, Ras/RAF/MEK/ERK und den β-Catenin-Signalweg. Das RASSF1A-Gen wird häufig durch eine abweichende Promotorhypermethylierung in der Mehrzahl von humanen Malignomen, einschließlich Lungen-, Magen-, Darm-, Blasen-, Kopf- und Halstumoren sowie gynäkologischen Tumoren, inaktiviert (Grawenda et al., 2015). Beim Ovarialkarzinom wurde die RASSF1A-Promotormethylierung in einigen Studien untersucht, jedoch keine signifikante Assoziation mit dem klinischen Ansprechen dokumentiert (Gloss et al., 2014). Eine genomweite Analyse zur Bestimmung von Methylierungsmustern bei Frauen mit Ovarialkarzinom zeigte, dass sich die Promotor Methylierung der drei Gene RASSF1A, CALCA und EP300 im Plasma von Patientinnen mit Ovarialkarzinom von den gesunden Plasmakontrollen unterschieden (Liggett et al., 2011). Das Resultat wird durch frühere Studien unterstützt, die häufiger RASSF1A-Promotormethylierungen im Ovarialkarzinom und selten im Plasma von Frauen mit benignen Tumoren identifizierten (Ibanez et al., 2004; Rathi et al., 2004; Ma et al., 2005; Teodoridis et al., 2005). Aktuell gibt es keine Studie, die eine Expression des RASSF1A-Gens im Gewebe und an ztDNA von korrespondierenden Plasmaproben untersucht, sowie die prognostische Bedeutung evaluiert hat.

Neben Untersuchungen der DNA werden miRNAs auch in biologischen Flüssigkeiten wie Serum oder Plasma, die als zirkulierende oder zellfreie miRNAs (zirmiRNAs) bekannt sind, zunehmend identifiziert und als nicht-invasive diagnostische Marker für verschiedene Tumorentitäten evaluiert.

2.7 Die Biogenese und Funktion der microRNAs

Neben Untersuchungen der DNA werden miRNAs auch in biologischen Flüssigkeiten wie Serum oder Plasma, die als zirkulierende oder zellfreie miRNAs (zirmiRNAs)

bekannt sind, zunehmend identifiziert und als nicht-invasive diagnostische Marker für verschiedene Tumorentitäten evaluiert.

MiRNAs sind endogen exprimierte, einzelsträngige, nicht-kodierende Ribonukleinsäuremoleküle (RNA), die eine Länge von 19-25 Nukleotiden besitzen (Nakamura et al., 2016). Sie werden anfänglich im Nukleus als lange primäre miRNAs, die sogenannte „primary-miRNA“, durch die RNA-Polymerase II transkribiert (Abb.2.11). Die primary-miRNA besitzt, ähnlich zur messengerRNA (mRNA), einen 5`7-Methyl-Guanosin-Cap und einen 3` poly(A)-Schwanz (Zeng et al., 2006).

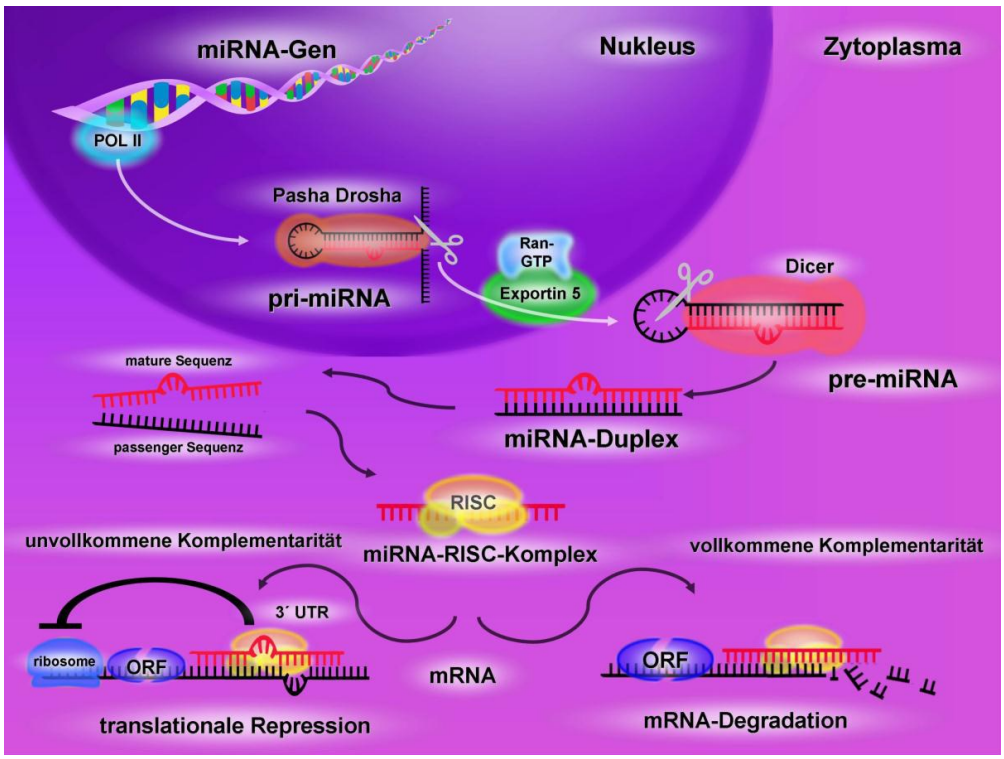

Abb.2.11 Prozessierung und Reifung der miRNA (Kuhlmann et al., 2012)

Die Bildung einer „Haarnadelstruktur“ mit einem doppelsträngigen Strang, terminalen Loop und flankierenden einzelsträngigen Bereichen wird aufgrund der Sequenzbeschaffenheit gebildet und als pri-miRNA bezeichnet. Für die Biogenese der miRNA sind die Enzyme DROSHA und DICER essentiell, indem Drosha mit seiner regulatorischen Untereinheit PASHA als Endonuklease die flankierenden RNA-Sequenzen der primary-RNA spaltet und dadurch das weitere Vorläufermolekül, die „precurser-miRNA“ entsteht. Alle anschließenden Reifungsschritte erfolgen im Zytoplasma der Zelle, indem als erster Schritt die Endonuklease DICER zum Einsatz kommt und die Entfernung der Loop-Sequenz aus der Haarnadelstruktur durchführt (Billy et al., 2001). Anschließend geht der „mature miRNA-Strang“ eine stabilisierende

Interaktion mit einem Ribonukleoproteinkomplex, dem „RNA-induced-silencing-complex“ (RISC-Komplex), ein. Der komplimentäre Strang, die „passenger RNA“, wird degradiert. Die mature miRNAs wirken als transkriptionelle Repressoren, indem sie durch die Bindung an der 3´untranslatierten Region (3`UTR) einer mRNA, die Genregulation inhibieren. Dieser Prozess ist auch als Konzept der negativen Genregulation bekannt. Das RISC-Protein verfügt über verschiedene Möglichkeiten, die Translation der mRNA zu verhindern, indem der Grad an Komplementarität zwischen der miRNA und dem 3´UTR der Ziel-mRNA eine essentielle Wirkung auf die Inhibition hat. Dabei gilt das Credo, je höher die Komplementarität, desto höher ist die Wahrscheinlichkeit, dass der RISC-Komplex die mRNA degradiert. Sollte die Komplementarität unvollkommen sein, so bindet der RISC-Komplex an die mRNA und supprimiert die Translation des Zielproteins, ohne einen Degradierungsvorgang zu starten (Esquela-Kerscher & Slack 2006). Alternativ kann es zu einer Deadenylierung der mRNA kommen (Wu et al., 2006).

2.8 MicroRNAs und das Ovarialkarzinom

Studien zur Analyse von microRNAs wurden beim Ovarialkarzinom sowohl am Gewebe, als auch im Plasma und Serum durchgeführt. Es konnte gezeigt werden, dass miR-141, miR-200a, miR-200b und miR-200c im Vergleich zu gesundem Ovarialgewebe signifikant überexprimiert waren. Im Gegensatz dazu lagen miR-125b1, miR-140, miR-145 und miR-199a herunterreguliert vor (Iorio et al., 2007). Weiterhin waren miR-200a, miR-200b und miR-429 prädiktiv für die Rezidiventwicklung und ein verkürztes OS (Hu et al., 2009), wobei eine dysregulierte let-7i Expression bei Patientinnen in der fortgeschrittenen Situation mit einem verkürztem PFS und der Platinresistenz korrelierte (Yang et al., 2008).

MiRNAs konnten nicht nur im Gewebe, Serum oder Plasma, sondern auch in der Brustmilch, im Speichel und Urin identifiziert werden (Nakamura et al., 2016). ZirmiRNA sind sehr stabil. Einerseits durch den pH-Wert und die Temperatur, andererseits durch RISC-assoziierte Proteine, sogenannte Argonaut-2 Proteine und extrazelluläre Membranvesikel, wie Exosomen oder Mikrovesikel (Valadi et al., 2007; Mitchell et al., 2008; Chen et al., 2008; Mause et al., 2010; Arroyo et al., 2011). Die Extraktion der zirmiRNA kann über die Zentrifugation und über Exosomenisolationskits verschiedener Firmen erfolgen (Nakamura et al., 2016). Die Detektion der miRNA und

zirmiRNA erfolgt entweder über NGS oder mittels Mikroarrays, ein technisches Verfahren, um die Aktivität und Identifikation von bestimmten Genen zu detektieren, um umfassende Profile von miRNA und zirmiRNA zu erhalten. Das diagnostische Potential der zirmiRNA konnte in wenigen Studien gezeigt werden. Die Serumanalyse von 180 Ovarialkarzinompatientinnen ergab, im Vergleich zu 66 Normalspendern, eine Herunterregulation von miR-25 und miR-93, während miR-7 und miR-429 überexprimiert vorlagen (Meng et al., 2015). Eine andere Studie detektierte im Plasma von 33 Ovarialpatientinnen eine erhöhte miR-200b-Expression (Kapetanakis et al., 2015).

Bezüglich der prognostischen Bedeutung korrelierten miR-21; miR-221; miR-23b; miR-29b; miR-21; miR-429; miR-141 und miR-200b signifikant mit einem verkürzten PFS oder OS (Xu et al., 2013; Hong et al., 2013; Vaksman et al., 2014; Meng et al., 2015; Gao et al., 2015; Kapetanakis et al., 2015). Im Gegensatz dazu war die verminderte Expression von let-7f; miR-1290; miR-200c und miR-145 signifikant mit einem verkürzten PFS oder OS assoziiert (Zheng et al., 2013; Shapira et al., 2014; Gao et al., 2015; Liang et al., 2015).

2.9 Zielsetzung

Trotz Fortschritten in der Behandlung des Ovarialkarzinoms beträgt die Rezidivrate etwa 75%. Weiterhin sind generell 15-20% der Patientinnen platinresistent, was bisher nur retrospektiv erkannt wird. Somit ist dem Ovarialkarzinom eine sehr schlechte Prognose zuzuordnen und die Etablierung neuer Biomarker zur Prognoseeinschätzung wäre äußerst wünschenswert. Daher ist es von großem Interesse, innovative primärtumor- bzw. blutbasierte Biomarkerkonzepte zu entwickeln, die uns Aufschluss über Prognose, Rezidiventwicklung bzw. Therapieansprechen geben oder im Sinne eines Therapie-Monitoring genutzt werden können. Da der Primärtumor nur zu Beginn der Erkrankung zur Verfügung steht, ist die sogenannte „Liquid Biopsy“ immer mehr im Fokus. Beim Ovarialkarzinom sind in diesem Zusammenhang DTZ, vor allen Dingen aber ZTZ sowie zirkulierende DNA und microRNAs vielversprechende Kandidaten.

Vorarbeiten:

Die Arbeitsgruppe um Frau Professorin Kasimir-Bauer konnte schon relevante Beiträge zur Etablierung neuer Biomarkerkonzepte für das Ovarialkarzinom publizieren und die Hypothese unterstützen, dass Patientinnenblut im Sinne einer „Real-Time-Liquid-Biopsy“ genutzt werden könnte. Im Hinblick auf DTZ und ZTZ wurde die schlechte Prognose der Patientinnen hinsichtlich des PFS und OS schon belegt (Wimberger et al., 2007, Aktas et al., 2011, Fehm et al., 2013). Insbesondere $ERCC1_{pos}$ ZTZ zu Beginn der Erkrankung konnten als prädiktiver Marker für ein verkürztes PFS, OS sowie eine klinische Platinresistenz identifiziert werden (Kuhlmann et al., 2014). Im Rahmen von DNA/RNA Untersuchungen wurde belegt, dass LOH (Loss of heterozygoity) von D6S1581, gemessen im Plasma von 63 primären Ovarialkarzinompatientinnen, signifikant mit dem PFS und OS korrelierte. Nach Therapie korrelierte LOH an D10S1765 mit der Tumorzellstreuung in das KM, was mit den zuvor erhobenen Daten im Primärtumor assoziierte (Kuhlmann et al., 2011, 2012). Weiterhin war die Arbeitsgruppe eine der ersten die belegten, dass zirkulierende, nichtkodierende RNU2-1f im Blut von Patientinnen mit Ovarialkarzinom im Vergleich zu Normalspendern signifikant erhöht war und persistierende Spiegel mit einer schlechten Prognose korrelierten (Kuhlmann et al., 2014).

Aufgabenstellung:

Basierend auf den oben genannten Daten wurden folgende Ziele für die Promotion formuliert:

Ziele:

1. Die erste Säule dieser Dissertation beinhaltete die Frage, ob persistierende DTZ oder DTZ mit Stammzellcharakter im KM den Verlauf der Erkrankung beeinflussen. Der Nachweis der DTZ unterliegt einer Standardmethode. Nach KM-Aspiration erfolgt eine Dichtegradientenzentrifugation mit anschließender Immunzytochemie unter Verwendung des Anti-Zytokeratin-Antikörper A45-B/B3, der die Zytokeratin-Heterodimere 8/18 und 8/19 detektiert. Hierbei handelt es sich um ein FAB-Fragment, um unspezifische Bindungen an Lymphozyten zu vermeiden. Der Nachweis erfolgt mit Neufuchsinrot (Abb.2.12). Während beim Mammakarzinom eine Reihe von Charakteristika der DTZ publiziert wurden, gibt es zum Ovarialkarzinom kaum Daten (Schindlbeck et al., 2016).

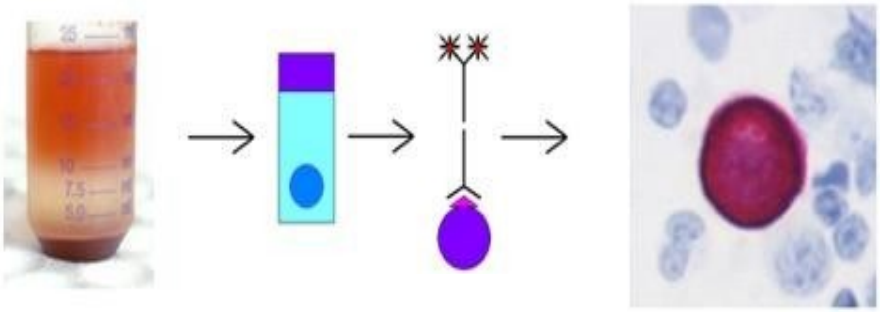

Abb.2.12 Schematische Darstellung d

Für den Nachweis des Stammzellcharakters der DTZ sollte eine vierfach Immunfluoreszenzfärbung etabliert werden, die folgende Marker beinhaltete: Zytokeratin (epithelialer Marker), LIN-28 und SOX-2 als Stammzellmarker, die mit dem Ovarialkarzinom assoziiert werden (Peng et al., 2010; Bareiss et al., 2013; Pham et al., 2013), CD34 zum Ausschluss von hämatopoeitischen Stammzellen und CD45 als Leukozytenmarker. Zur Etablierung der Methoden wurden die ovariellen Zelllinien OVCAR-3 sowie die Leukämiezelllinie Kasumi-1 verwendet. Die Methode wurde dann bei KM-Präparaten von Ovarialkarzinompatientinnen vor und nach Therapie angewendet.

2. Da KM Punktionen sehr invasiv sind und besonders nach Therapie häufig nicht akzeptiert werden, stand für weitere Fragestellungen das Blut als „Liquid Biopsy“ im

Fokus. Hierzu zählte die Fragestellung, ob ERCC1-positive, persistierende ZTZ sowie ZTZ in EMT die schlechte Prognose des Ovarialkarzinoms begleiten. Zur Selektion und Detektion der ZTZ verwendet das Labor der Frauenklinik den AdnaTest *OvarianCancer* (Abb.2.13), ein immunmagnetisches Selektionsverfahren mit anschließender Multiplex-RT-PCR.

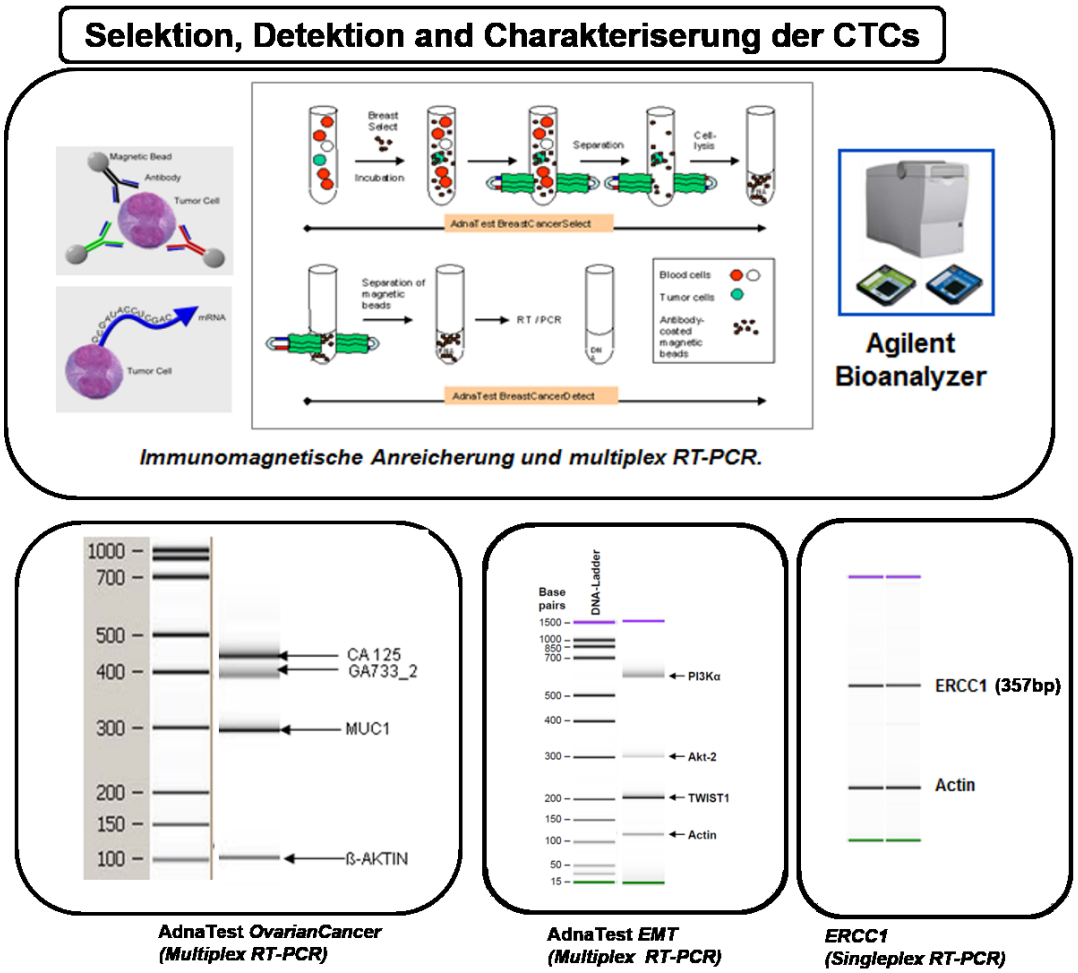

Abb.2.13 Schematische Darstellung der immunmagnetischen der ZTZ sowie deren Nachweis mittels PCR (Quelle Qiagen)

Der AdnaTest der Firma QIAGEN basiert auf einer immunmagnetischen Zellselektion mit den Gewebeassoziierten Antikörpern (EpCAM, MUC). Im ersten Schritt werden die Zellen angereichert und lysiert, danach die mRNA isoliert, gefolgt von einer Reversen Transkription und der Auswertung mittels Kapillarelektrophorese (BioAnalyzer). In zwei Testverfahren können durch Multiplex RT-PCR folgende Marker nachgewiesen werden, wobei einzelne Gene von Interesse mittels Singleplex PCR bestimmt werden können.

1. AdnaTest *OvarianCancer* (CA-125, EpCAM, MUC)
   ERCC1 wird als single-plex PCR durchgeführt
2. AdnaTest *EMT* (PI3Kα, AKT-2, TWIST)

Im Vordergrund der Dissertation stand die Rekrutierung von Blut vor und nach abgeschlossener Platintherapie, um explizit die Rolle von $ERCC1_{pos}$ ZTZ nach Therapie sowie deren Persistenz zu evaluieren. Die Rolle von ZTZ in EMT beim Ovarialkarzinom wurde bisher noch von keiner Arbeitsgruppe untersucht. Bekannt war bisher nur, dass Twist als Transkriptionsfaktor im Gewebe der Ovarialkarzinome von Bedeutung ist. (Kajiyama et al., 2006; Hosono et al., 2007).

3. Zur Evaluierung von small RNA Profilen sollte im Plasma/Serum von Patientinnen mit Ovarialkarzinom vor Therapie zunächst eine Methode etabliert/optimiert werden, die mittels NGS das Profil platinsensitiver und platinresistenter Patientinnen darstellt, sodass im Anschluss ausgewählte small RNA Kandidaten durch RT-qPCR validiert werden können. Dieses Projekt wurde in Kooperation mit Herrn Dr. Kuhlmann aus der Frauenklinik in Dresden und Herrn Dr. Michael Reuter aus dem Fraunhofer Institut in Leipzig durchgeführt.

Nebenziel:

Mit der Arbeitsgruppe von Frau Prof. Dr. Evi Lianidou in Athen besteht eine langjährige Kooperation. Im Rahmen der Thematik blutbasierter Biomarker stand die Methylierung zirkulierender Tumor-DNA im Vordergrund, wobei auch Vergleichsanalysen im Primärtumor durchgeführt wurden. Hier wurde eine entsprechende Validierungskohorte Tumor/Plasma aus der Frauenklinik zur Verfügung gestellt.

3 Publikationen

Analysis of disseminated tumor cells before and after platinum based chemotherapy in primary ovarian cancer. Do stem cell like cells predict prognosis?

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Key words:
Disseminated tumor cells, bone marrow, stem cells, primary ovarian cancer

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Abstract

Background: We recently reported that the presence of disseminated tumor cells (DTCs) in the bone marrow (BM) of primary ovarian cancer patients (POC pts) correlated with reduced progression free survival (PFS) and overall survival (OS). Here we analyzed whether the negative prognostic influence was related to DTC persistence after *platinum based chemotherapy* and/or due to DTCs associated with stem cell character.

Patients and Methods: 79 POC pts were studied for DTCs before therapy (BT) and after therapy (AT) using immunocytochemistry. Eight pts harboring at least five DTCs AT were further analyzed on two additional slides by four-fold immunofluorescence staining for DAPI, Cytokeratin (CK), SOX-2 or LIN-28, CD45 and CD34 (Cy5). A stem-like tumor cell was classified as $Dapi_{pos}$, $CD45_{neg}$, $CD34_{neg}$, $SOX\text{-}2_{pos}$/$LIN\text{-}28_{pos}$ and CK_{pos} or CK_{neg}.

Results: DTCs were detected in 33/79 pts (42%) before and in 32/79 pts (41%) AT. Persistent DTCs were found in 13 pts, 20 pts were only positive BT, 19 pts AT and 27 pts had no DTCs. Whereas the presence of DTCs BT significantly correlated with reduced OS (p= 0.02), pts initially DTC_{neg} BT but DTC_{pos} AT had a significantly shorter PFS (p=0.03). DTC persistence resulted in a shorter PFS and OS reaching borderline significance (p=0.06; p=0.07). LIN-28-and SOX-2 positive cells were detected in all eight pts AT.

Conclusion: Stem cell associated proteins are expressed in DTCs that are present AT and their presence seem to be correlated with a worse outcome. Additional therapeutic regimens may be necessary to eliminate these cells.

Introduction

Ovarian cancer is the fifth leading cause of all cancer related deaths in Europe and the United States and most tumors are diagnosed in an advanced stage with poor prognosis for the patients (1). Conventional therapy is based on an initial debulking surgery aiming at macroscopic complete resection combined with subsequent platinum- and paclitaxel -based chemotherapy (2). Postoperative residual tumor is one of the most important prognostic factors in advanced ovarian cancer (3,4,5).

It is hypothesized that cancer malignancy and metastasis are driven by a small subgroup of highly tumorigenic cells within the tumor, called metastasis initiating cells (MIC). These cells have the ability to self-renew, enhance tumorigenesis and are often found to be drug resistant (6). The presence of such a small population, often referred to as cancer stem cells (CSC), has been confirmed in ovarian cancer cell lines as well as in tumor tissue (7,8). Their amount is increased in chemotherapy resistant ovarian cancer cell lines (7) and they are believed to contribute to an aggressive behavior of epithelial ovarian cancer (9). The pluripotency associated stem cell factors SOX2 (sry related) and LIN-28 have been found to be expressed in ovarian cancer cell lines and tissue (10,11,12). Bareiss et al., showed that SOX2 expression is a CSC marker in serous ovarian carcinomas (SOC) and can induce CSC properties (11). In addition, SOX2 was reported to enhance migration and invasion of ovarian cancer cells (13). Importantly, SOX2 overexpression was shown to be a poor prognostic marker in ovarian cancer (14) and also shown to be involved in taxane resistance (15,16).

In ovarian cancer, the primary tumor usually metastasizes to the peritoneum, but a variety of studies including ours indicate that tumor cells frequently disseminate into the bone marrow (BM). Disseminated tumor cells (DTCs) in the BM are detected in 20% to 60% of cases before the onset of platinum-based chemotherapy depending on the method of detection used. Their prognostic relevance with regard to reduced progression free survival (PFS) and overall survival (OS) has previously been demonstrated (17,18,19,20,21). In addition, we demonstrated that patients with a marked increase of DTCs after platinum-based chemotherapy showed a significantly reduced PFS (22).

Based on the studies mentioned above, there is increasing evidence that DTCs could reflect cancer progression. Thus, DTCs could be used as novel targets for additional therapeutic strategies. In this study, we analyzed whether our previously reported negative prognostic influence of DTCs with regard to reduced PFS and OS 1) was related to the persistence of DTCs after platinum based chemotherapy and/or 2) might

have arisen from a cellular phenotype showing stem cell characteristics.

Results

Detection of DTCs

Before therapy (BT), DTCs were detected in 33/79 patients (42%) with a median number of 4 DTCs (range 1-37). After therapy (AT), 32/79 patients (41%) were positive for DTCs (median cell number of 8 cells (range 1-100) (Table 1). DTCs were found in 13 patients BT and AT, in 20 patients only BT and in 19 patients only AT, respectively. DTCs were not detected in samples taken BT or AT from 27 patients (Table 2).

Prognostic significance of DTCs

After a median follow up time of 62 months (range 10-128 months), 44 patients (56%) were still alive and 33 patients (42%) had died. The median follow-up time for PFS was 15 months (range 4-87 months) resulting in 53 (67%) relapses while 25 patients (32%) had no relapse (Table 1). The presence of DTCs BT significantly correlated with reduced OS (p=0.02) and patients initially DTC_{neg} BT but DTC_{pos} AT had a significant shorter PFS (p=0.03) (Table 2 and Fig.1). The persistence of DTCs resulted in a shorter PFS and OS reaching borderline significance (p=0.06; p=0.07).

Evaluation of Lin28- and Sox-2-positive cells

Staining of patient samples is shown in Fig. 2-5. Controls are shown in Supplementary Fig. 1-3. A DTC was classified as a stem-like tumor cell if it had the following staining characteristics: $Dapi_{pos}$, $CD45_{neg}$, $CD34_{neg}$, $SOX\text{-}2_{pos}$/$LIN\text{-}28_{pos}$ and CK_{pos} or CK_{neg} (Fig. 2-5). The Kasumi cell line was used to establish CD34 expression (Suppl. Fig. 1) and BM samples from healthy donor patients for CD34- and CD45-expression (Suppl. Fig. 2 and 3).

Detection of LIN-28- and SOX-2-positive cells

DTCs from 10 patients were analyzed BT and AT for SOX-2 and LIN-28 positive cells AT (Table 3; columns 1 and 2; Suppl. Table 1). 8/10 patients had at least five DTCs as detected by immunocytochemistry using A45/B-B3. In addition, 2/10 patients (patient 2 and 5) were DTC_{neg} AT but DTC_{pos} BT. As apparent from Table 3, AT CK_{pos}/$LIN\text{-}28_{pos}$ cells were detected in 9/10 patients [median 2 cells (range 1-5)] and CK_{neg}/$LIN\text{-}28_{pos}$ cells in 7/10 patients [median 3 cells (range 1-11)], respectively. CK_{pos}/$SOX\text{-}2_{pos}$ cells were detected in 6/8 patients [median 2 cells (range-0-4)] and CK_{neg}/$SOX\text{-}2_{pos}$ cells

were found in 7/8 patients [median 4 cells (range 1-11)]. Patients two and five, who were characterized as DTC_{neg} AT by immunocytochemistry but were positive BT (37 and 6 DTCs, respectively) were included in our analysis for stem cell- associated markers. Interestingly, these two patients harbored 1-2 LIN-28_{pos} and SOX-2_{pos} cells in their BM AT. Thus, we evaluated LIN-28-/SOX-2-positive cells BT in cases vice versa, DTC_{neg} BT but DTC_{pos} AT (patients 1, 3 and 4) as well as in patient number 6 with persistent DTCs. As shown in Table 3, in patients who switched initially DTC_{neg} before but becoming DTC_{pos} AT (patients 1, 3 and 4), in patients who switched from being DTC_{pos} before but becoming DTC_{neg} AT (patients 2 and 5) as well as in patient number 6 with persistent DTCs (patient 6) a few LIN-28 as well as SOX-2-positive cells were present in BM even BT.

Discussion

To the best of our knowledge, this is the first study showing that DTCs, present after platinum based chemotherapy in primary ovarian cancer patients show stem cell characteristics. Furthermore, although p values reached borderline significance, these cells might be associated with worse outcome which finally has to be proven in a bigger patient cohort.

The rate of DTC detection in primary ovarian cancer before the administration of platinum-based chemotherapy has been reported to be 20% to 60%, depending on the method of detection used. Furthermore, their presence has been associated with worse outcome (17,18,19,20,21,22). The lack of significant correlation between DTCs and clinical outcome reported by other investigators may be due to their use of different antibodies for detection of DTCs (23,24).

In this study, DTCs were present in the BM AT in 41% of the patients which is in accordance with our earlier data which also demonstrated that DTCs, still present AT, were non-apoptotic and their marked increase was associated with a significantly reduced PFS (22). These findings suggest that the BM may to be a temporary homing site for isolated tumor cells, where they can persist and potentially induce recurrence of the disease. Analyzing 79 patients BT and AT, we confirm the negative prognostic influence of DTC detection with regard to OS (20). We observed persistent DTCs in 16% of the patients which was associated with a shorter PFS and OS, reaching borderline significance. Interestingly, 24% of the patients that were initially characterized as DTC_{neg} BT and converted to DTC_{pos} AT had a significantly shorter PFS. The negative prognostic influence of these cells could be in alliance with a

currently discussed hypothesis that some DTCs may have cancer stem cell features and may be the active source of metastatic spread in primary tumors, in addition to resistance to various chemotherapeutic agents and radiotherapy (25). Two studies have confirmed a putative stem cell phenotype among DTCs in breast cancer patients (26,27). Here, we demonstrate that presence of DTCs that persist AT express the stem cell markers Lin-28 and/or SOX-2. We show that stem cell-like cells were present before the administration of chemotherapy. These findings may explain the significantly shorter PFS of patients who changed from DTC_{neg} BT to DTC_{pos} AT. In addition, patients characterized as DTC_{neg} AT also harbored some Lin-28 and/or SOX-2 positive cells in their BM which may be responsible for a worse outcome. Until now, tumor stem cells have only been analyzed in ovarian tumor tissue, but not in DTCs. In this regard, previous studies have shown that LIN-28, SOX-2 as well as OCT-4 play a major role in carcinogenesis (10,11,12). Wang et al., reported that SOX-2 targets SRC Kinase, a non-receptor tyrosine kinase that increases cell migration, invasion and adhesion of serous ovarian carcinoma cells (13). Inhibition of either LIN-28 or Oct-4 expression decreases cell viability. The combined repression of both LIN-28 and Oct-4 results in synergistic inhibition of cancer cell growth and survival of ovarian cancer cell lines (9). Expression of SOX-2 has been investigated by immunohistochemistry analysis of normal ovarian epithelial, serous and mucinous cystadenoma and cystadenomacarcinoma specimens (28) and LIN-28 was overexpressed in different epithelial tumors including breast, lung, colon and ovarian cancer (29). Furthermore, SOX-2 may be crucial for the development of chemotherapy resistance. Yang et al., analyzed SOX-2 expression in clinical tissue samples and ovarian cancer cell lines using immunohistochemistry and real-time PCR and demonstrated that SOX-2 was overexpressed in paclitaxel-resistant cells (30). In ovarian cancer patients receiving taxanes, expression of SOX-2 was shown to be correlated with chemotherapy resistance and a shorter PFS whereas patients receiving non-taxane based chemotherapy showed no significant response influence (31). Since the patients in our study have also received a combined therapy with paclitaxel and carboplatin, it may be possible that SOX-2 expression on DTCs was associated with chemotherapy resistance. However, mechanisms associated with chemotherapy resistance in ovarian cancer still remain unclear. Functionally, primary platinum-resistance, defined as platinum-free treatment interval of less than 6 months observed in up to 20% of ovarian cancer patients, can be the result of either increased tolerance towards DNA-platinum-adducts or enhanced DNA-repair capacity of tumor cells (32,33,34). In this

context, we recently demonstrated that ERCC1$_{pos}$ (excision-repair cross-complementing rodent repair deficiency, complementation group 1 nuclease) circulating tumor cells (CTCs) constituted an independent predictor, not only for OS but also for PFS in our ovarian cancer patients. Most interestingly, the presence of ERCC1$_{pos}$ CTC at primary diagnosis was an independent predictor for platinum-resistance whereas ERCC1-expression in the corresponding primary tumor tissue predicted neither platinum-resistance, nor prognosis (35). Consequently, assuming that CTCs must be spread into the circulation from existing pools in secondary organs, e.g. the BM, one might speculate the ERCC1$_{pos}$ DTCs also exist and contribute to platinum resistance. Interestingly, DTCs present BT in our patients significantly correlated with clinical platinum resistance (data not shown).

In this study, we also detected CK$_{pos}$/SOX-2$_{pos}$ (LIN-28$_{pos}$) as well as CK$_{neg}$/SOX-2$_{pos}$ (LIN-28$_{pos}$) cells in all patients. We assume that two different cell types with expression of stem cell associated proteins may have been detected. It has been described that tumor cells undergo phenotypic changes, known as epithelial-mesenchymal transition (EMT), which allow them to migrate to sites of metastasis without being eliminated by conventional treatment (36). Thus, CK$_{neg}$/SOX-2$_{pos}$ (Lin-28$_{pos}$) cells might result from EMT while the CK$_{pos}$/SOX-2$_{pos}$ (LIN-28$_{pos}$) epithelial phenotype may have remained unchanged.

Taking all these considerations into account, additional therapeutic strategies will be required to target signaling pathways concerning CSC. These studies will include mTOR inhibitors, acting downstream of the PI3K/AKT pathway (37), salinomycin (38) or a new synthetic curcumin analogue against ALDH1 and GSK-3ß (39). Finally, approaching the tumor microenvironment, such as interrupting the immune cells and cytokines (e.g. IL-6, IL-8) as well as the immune checkpoints (PD1/PDL1) may provide additional new tools for immunological killing of cancer stem cells (40,41,42)

Conclusion and limitation of the study: The cohort of our patients is probably too small to draw the final conclusion that a significant selection of stem cell marker-positive DTCs occurs during chemotherapy. Consequently, the results presented here should be viewed as a “proof of principle”, that DTCS with stem cell characteristics exist among DTCs that are present BT and persist AT. To the best of our knowledge, we are the only group that has a collection of BM cells harvested from primary ovarian cancer patients AT. In this regard, 79 paired samples from patients who consented to allow collection of their BM under local anesthesia AT for research purposes is a unique collection and would be difficult to achieve high patient numbers. Furthermore,

based on the number of residual slides and methodological requirements, we only analyzed two stem cell markers. Ongoing studies will include other stem cell markers, such as OCT4 as well as resistance marker to finally elucidate the prognostic relevance of these cells.

Patients and Methods

Patient characteristics

79 patients with primary ovarian cancer who presented at the Department and Gynecology and Obstetrics, University Hospital Essen between February 2004 and January 2010 were included in this analysis. Patient characteristics are documented in Table 1. The mean age was 60 years (range 26-86 years), the median follow-up time was 62 months (10-128 months) for OS and 15 months (4-87 months) for PFS. Written Informed consent was obtained from all patients and the study was approved by the Local Ethics Committee (05-2870). Tumors were classified according to the WHO classification of tumors of the female genital tract. Grading was conducted using the grading system proposed by Silverberg (43) and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique (FIGO 2009). The entire study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping was performed and in addition to pelvic and para-aortic lymphadenectomy, if macroscopic complete resection was achieved. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if complete tumor resection was achieved intraperitoneally following actual guidelines (www.ago-ovar.de). All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m2. Tumors were clinically defined as platinum-resistant if they recurred within six months after the completion of platinum-based chemotherapy.

Cell lines

The human ovarian cancer cell line OVCAR-3 and the Kasumi-1 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 containing 10% (20% for Kasumi-1) fetal calf serum and 1% (100 U/ml) Penicillin-Streptomycin (Gibco™ by Thermo Fisher Scientific, Waltham MA, US) . Cells were grown at 37 °C in a humidified atmosphere with 5% CO2.

Detection of DTCs

Between 10 and 20 ml BM were aspirated from the anterior iliac crests and processed within 24 hours. DTC selection and detection was performed based on the recommendations for standardized tumor cell detection (44). Details of the staining procedure and cell detection have been described elsewhere (22). Briefly, BM cells were isolated from heparinized BM (5000 U/ml BM) by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400 x g for 30 min. Slides were analyzed for DTCs by immunocytochemistry using the pan-cytokeratin antibody A45-B/B3. Microscopic evaluation of the slides (1 x 10^6 mononuclear cells per slide) was carried out using the ARIOL system (Applied Imaging) according to the ISHAGE evaluation criteria (45).

Detection of LIN-28- and SOX-2-positive cells

LIN-28- and SOX-2 positive cells were analyzed separately on additional slides of the same patients harboring at least five DTCs as detected by immunocytochemistry using the A45B-B3. Four-fold immunofluorescence staining was established using the OVCAR-3 cell line spiked into blood of healthy donors. Since CD34-positive normal hematopoietic stem cells comprise 1.5% of marrow mononuclear cells (46), we included CD34 in our analysis to exclude false-positive results. CD34 was analysed using the Kasumi-1 cell line since BM of healthy donors was difficult to obtain and only available from one donor (Suppl. Fig. 1).

Slides were fixed with 4% Paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X-100 for 15 min and subsequently washed with TBS and Triton-X-100 three times for five min. Slides were stained with SOX-2 (Anti-human SOX-2, 1:50, R&D Systems, USA), LIN-28 (LIN-28, 1:350, Rabbit polyclonal ab46020, Abcam, UK), C11 (anti-PAN-Cytokeratin, 1:400, FITC-labelled, GeneTex, USA), CD34 (Alexa Flour anti-human 647, 1:100,Cy5 labelled, Biolegend, USA) and CD45 (Alexa Fluor 647;sc1178 Santa Cruz, USA) incubated in a wet chamber for one hour at room temperature. Subsequently, slides were incubated with DAPI [pre diluted in Phosphate Buffered Saline (1:250) and further diluted in AB diluent (1:20, Dako, Germany)], and TRITC-labelled donkey anti-goat 594 (SOX-2), donkey anti-rabbit 594 (LIN-28) (both1:100, Invitrogen, USA) under the same conditions followed by three washing steps for five minutes. Moreover, negative controls for primary antibodies were prepared by staining the spiked OVCAR-3 cell line with the secondary antibodies for 30 minutes under the same conditions. The slides were mounted with Dako fluorescent mounting medium

s3023 and a coverslip and dried overnight in a cooling chamber. Counting was performed visually, using an immunofluorescence microscope (Axioplan 2 Imaging Zeiss Germany, Metasystems) and Isis Fish imaging system V5.3 (Meta Systems, Germany) at a magnification of 40x or 63x.

Statistical Analysis

Survival analysis was performed by using Winstat (2012.1) an upgrade of Microsoft Excel. Survival intervals were screened from the time of BM aspiration at first diagnosis to the time of death or first time of relapse, defined as either local recurrence or distant metastasis. Kaplan-Meier curves were established using the log-rank test to evaluate univariate significance of the parameters.

Tab.1. Patient characteristics at the time of primary diagnosis.

Total	**79**
Age	median 60 years, (26-86)
FIGO stage	
I-II	21 (26%)
III	48 (61%)
IV	10 (13%)
Nodal status	
N_o	32 (40.5%)
N_1	32 (40.5%)
N_x	10 (19%)
Grading	
I-II	44 (56%)
III	33 (42%)
Unknown	2 (2%)
Residual tumor	
Macroscopic	
Complete resection	49 (62%)
Any residual tumor	30 (38%)
Histologic type	
Serous	47 (60%)
Mucinous	9 (11%)
Other	23 (29%)
DTC	
Before therapy	33 (42%)
After therapy	32 (41%)Survival

PFS[1]	median 15 months, (4-87 months)
OS[2]	median 62 months, (10-128 months)
Alive	44 (56%)
Dead	33 (42%)
Unknown	2 (2%)
Recurrence	
No relapse	25 (32%)
Relapse	53 (67%)
Unknown	1 (1%)
Platinum resistance	
Platinum sensitive	60 (76%)
Platinum resistant	19 (24%)

[1]PFS: progression-free survival, [2]OS: overall survival

Tab.2. Prognostic significance of DTCs before and after therapy with regard to PFS and OS.

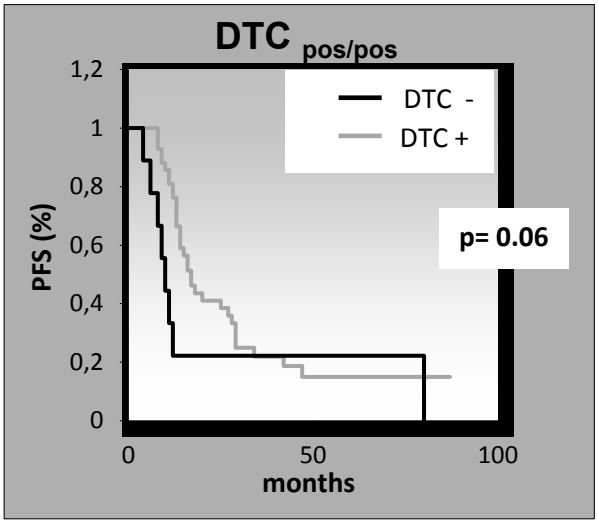
Status	Number of patients (n)	PFS (p-value)	OS (p-value)
Total	79		
DTC_{pos} before therapy	33 (42%)	0.06	**0.02**
DTC_{pos} after therapy	32 (41%)	0.35	0.98
DTC_{pos}/DTC_{pos}	13 (16%)	0.06	0.07
DTC_{neg}/DTC_{neg}	27 (34%)	0.77	0.31
DTC_{pos}/DTC_{neg}	20 (25%)	0.46	0.25
DTC_{neg}/DTC_{pos}	19 (24%)	**0.03**	0.18

Tab.3. Distribution of DTCs and LIN-28-/SOX-2-positive cells before and after therapy.

			before therapy				after therapy			
Patients	**DTZ_{pos} before therapy (A45-B/B3)**	**DTZ_{pos} after therapy (AF45-B/B3)**	**Ck_{pos}/LIN-28_{pos}**	**Ck_{neg}/LIN28_{pos}**	**Ck_{pos}/SOX-2_{pos}**	**Ck_{neg}/SOX-2_{pos}**	**Ck_{pos}/LIN-28_{pos}**	**Ck_{neg}/LIN-28_{pos}**	**Ck_{pos}/SOX-2_{pos}**	**Ck_{neg}/SOX-2_{pos}**
1	0	14	25	5	24	2	4	0	3	4
2	37	0	2	0	4	1	0	1	2	0
3	0	11	8	2	5	1	2	0	0	1
4	0	15	2	1	1	1	2	1	nsa	nsa
5	6	0	1	1	2	1	1	0	2	1
6	28	18	nsa	nsa	2	3	3	7	4	11
7	0	5	nsa	nsa	nsa	nsa	1	3	0	2
8	0	100	nsa	nsa	nsa	nsa	5	9	1	2
9	1	35	nsa	nsa	nsa	nsa	2	1	nsa	nsa
10	0	10	nsa	nsa	nsa	nsa	3	11	2	9

nsa: no slides available

A


DTC pos/pos
DTC -
DTC +
p= 0.06
PFS (%)
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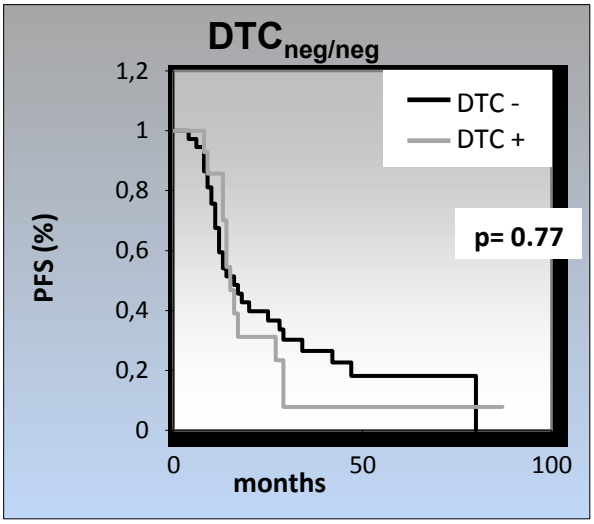
1
0,8
0,6
0,4
0,2
0
0
50
100
months

B


DTC neg/neg
DTC -
DTC +
p= 0.77
PFS (%)
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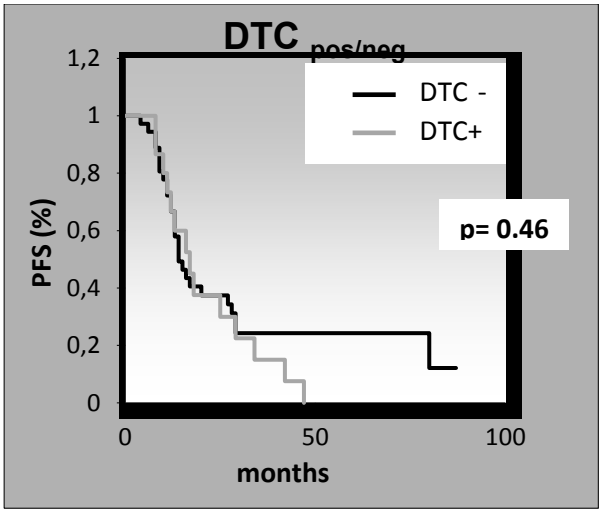
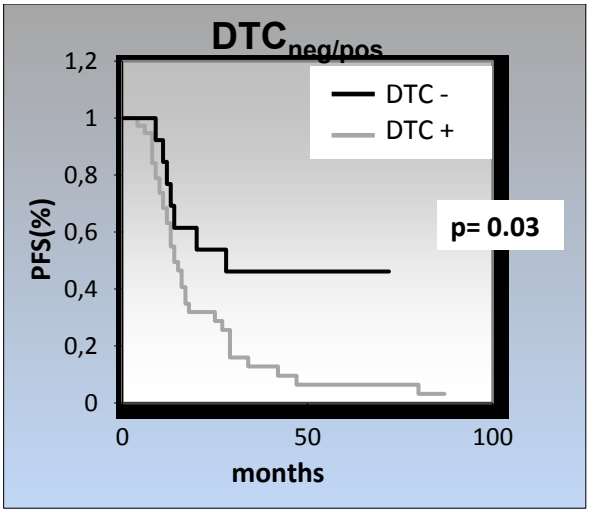
1
0,8
0,6
0,4
0,2
0
0
50
100
months

C


DTC pos/neg
DTC -
DTC+
p= 0.46
PFS (%)
1,2
1
0,8
0,6
0,4
0,2
0
0
50
100
months

D


DTC neg/pos
DTC -
DTC +
p= 0.03
PFS(%)
1,2
1
0,8
0,6
0,4
0,2
0
0
50
100
months

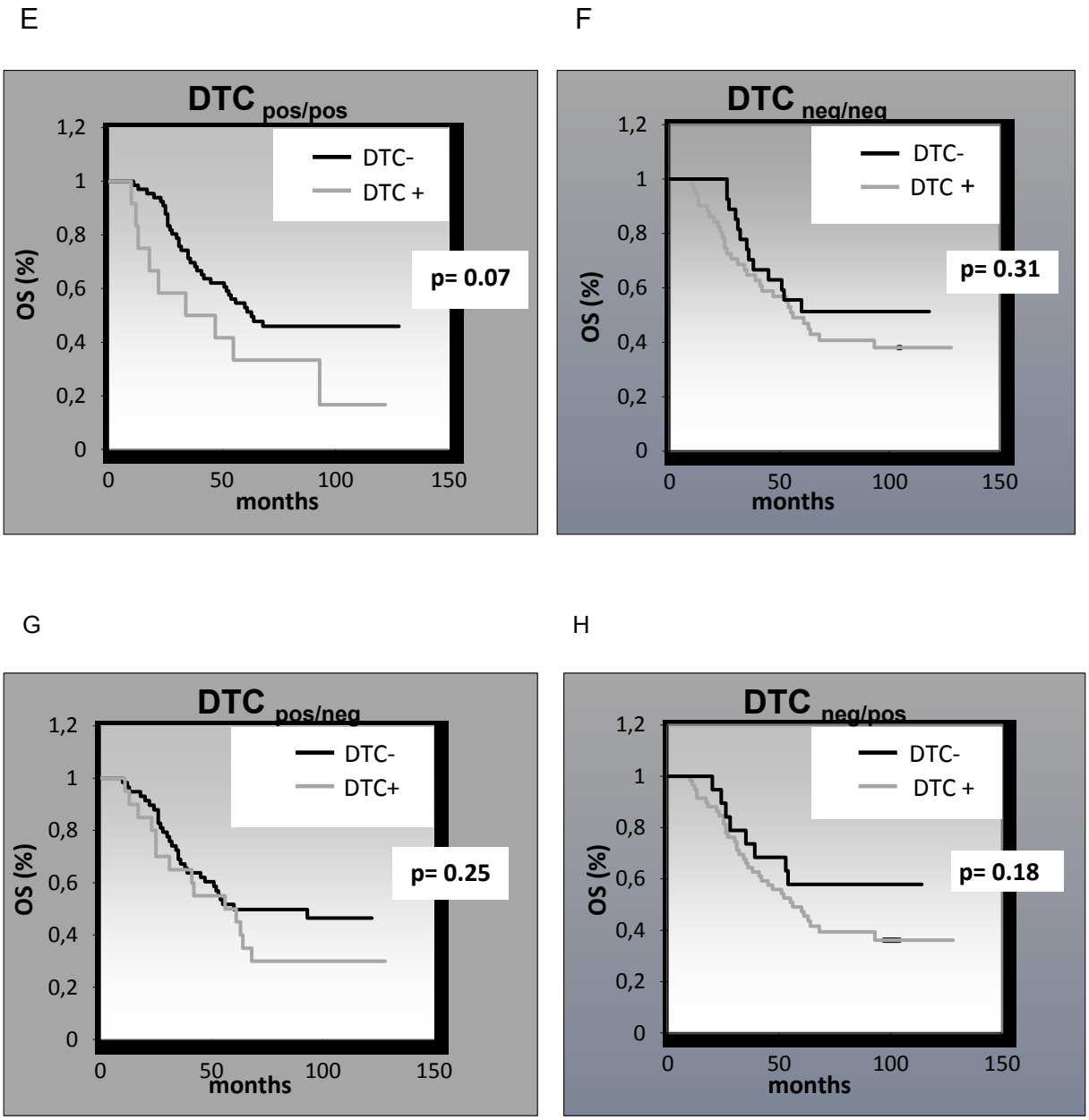


Fig.1. Kaplan-Meier analysis for the correlation of PFS (A-D) and OS (E-H) with DTC detection. Patients initially DTC_{neg} before therapy but DTC_{pos} after therapy had a significant shorter PFS (p=0.03) (Fig. 1D).

A. PFS	**$DTC_{pos/pos}$**	**E.**	**OS** **$DTC_{pos/pos}$**
B. PFS	**$DTC_{neg/neg}$**	**F.**	**OS** **$DTC_{neg/neg}$**
C. PFS	**$DTC_{pos/neg}$**	**G.**	**OS** **$DTC_{pos/neg}$**
D. PFS	**$DTC_{neg/pos}$**	**H.**	**OS** **$DTC_{neg/pos}$**

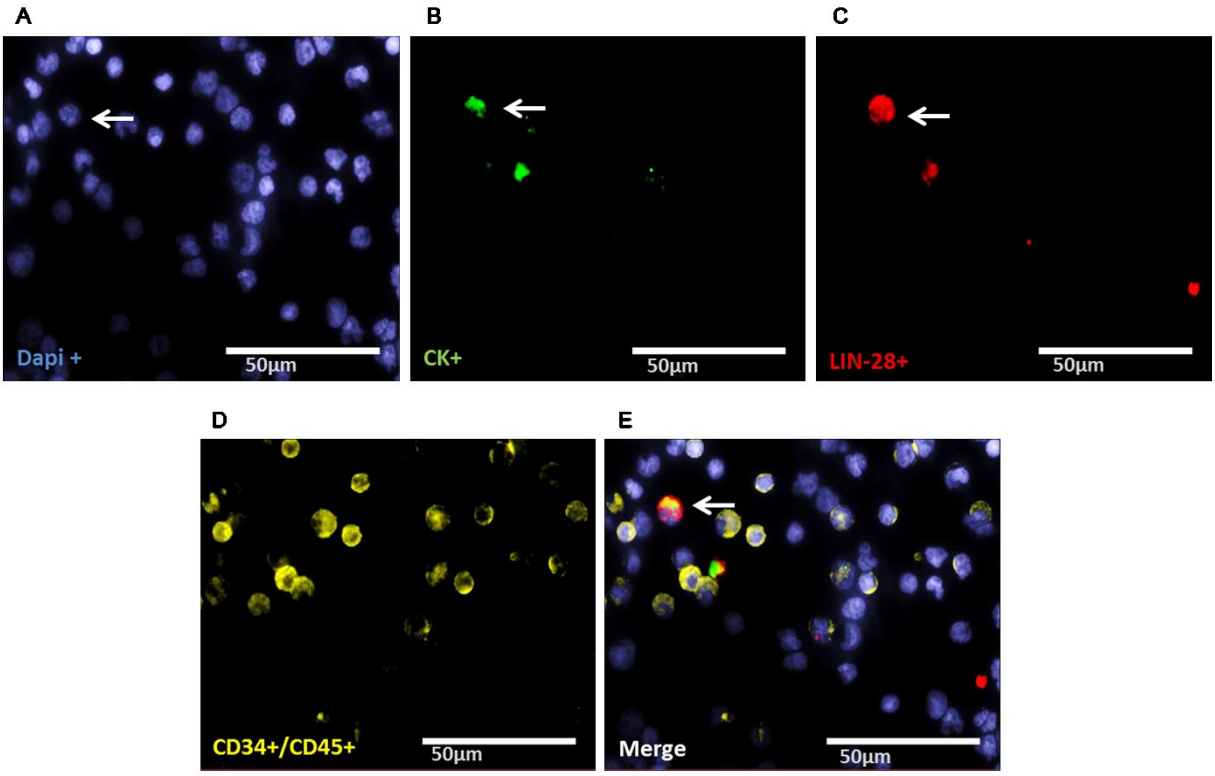


Fig.2. Representative four-fold immunofluorescence staining for CK_{pos}/LIN-28_{pos} cells after therapy of patient No1.

A) Cell nuclei were stained with Dapi. B) Indicates a CK_{pos} cell. C) Alludes a cell with LIN-28_{pos} phenotype. D) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. E) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{pos}, LIN-28_{pos}, $CD34_{neg}$ and $CD45_{neg}$, magnification at 63x.

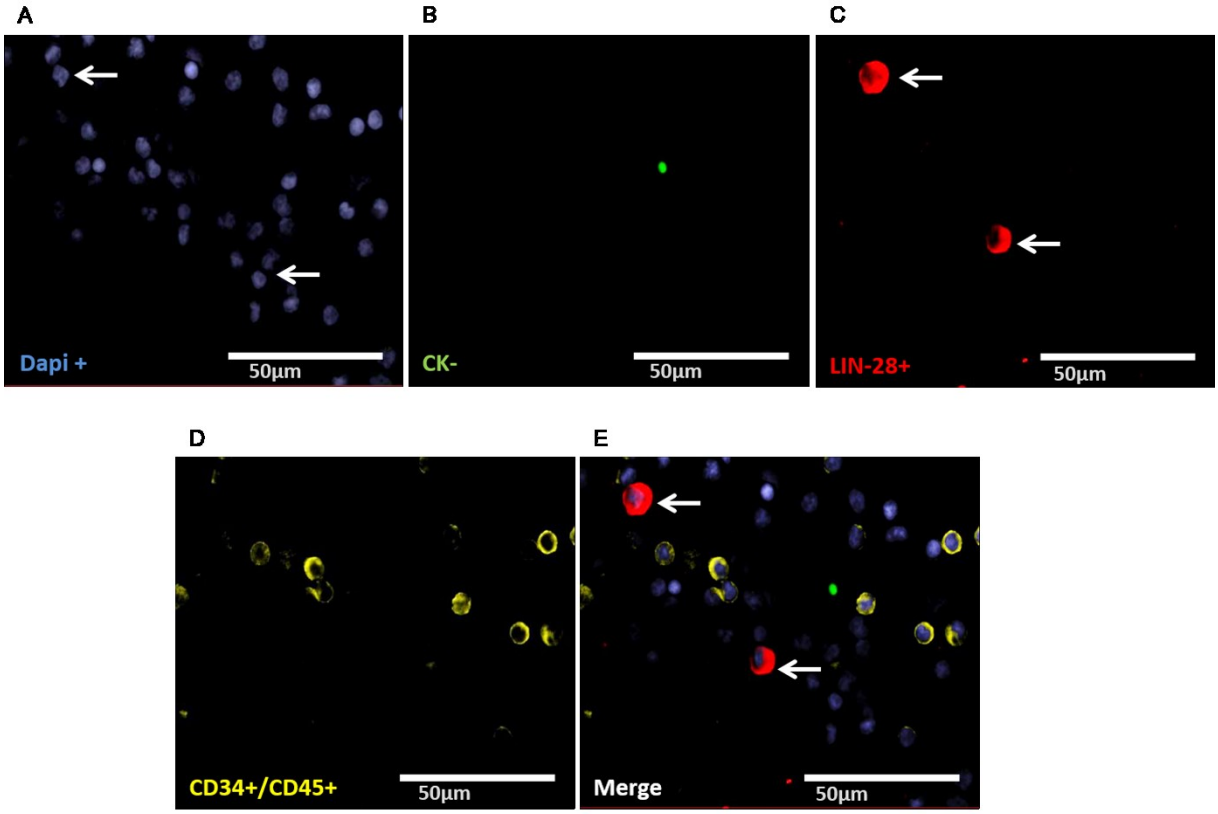


Fig.3. Representative four-fold immunofluorescence staining for CK_{neg}/LIN-28_{pos} cells after therapy of patient No.1

A) Cell nuclei were stained with Dapi. B) Indicates a CK_{neg} cell.
C) Alludes a cell with LIN-28_{pos} phenotype. D) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. E) Indicates a merge of two DTCs with the phenotype $Dapi_{pos}$, CK_{neg}, LIN-28_{pos}, $CD34_{neg}$ and/or $CD45_{neg}$, magnification at 63x.

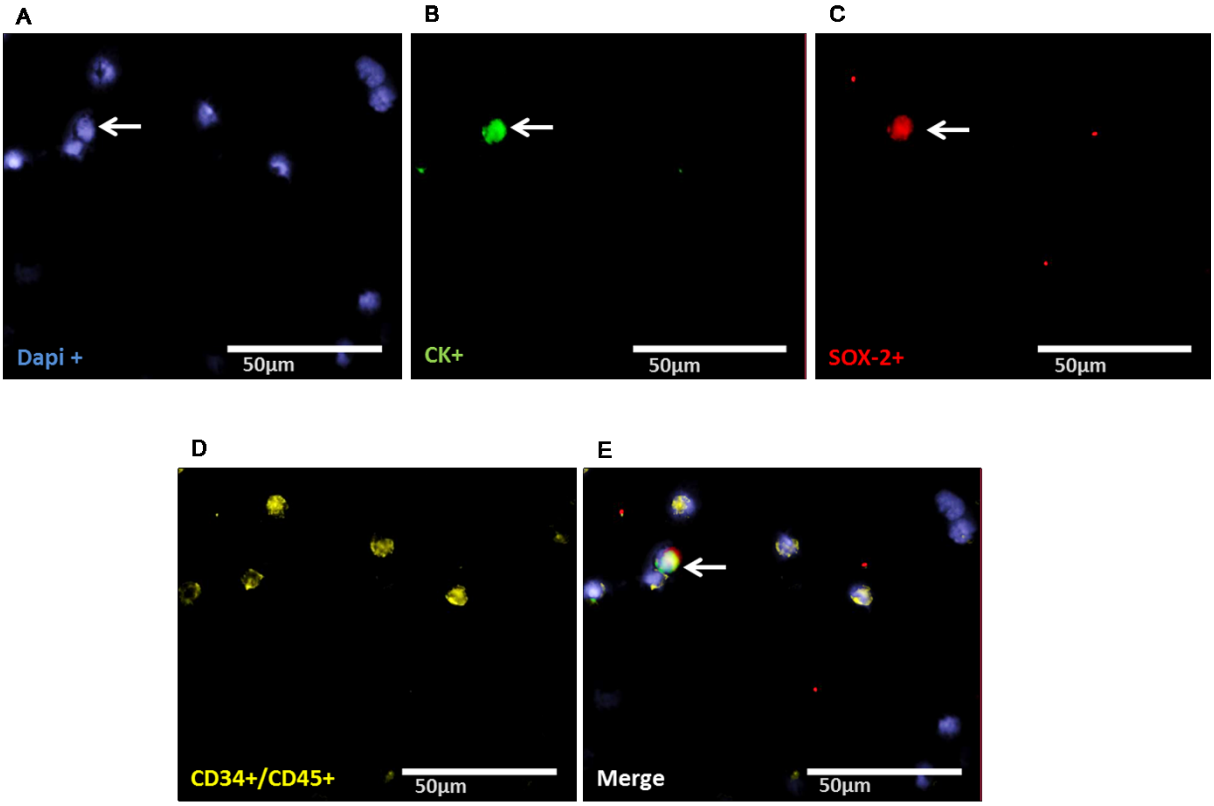


Fig.4. Representative four-fold immunofluorescence staining for CK_{pos}/SOX-2_{pos} cells after therapy of patient No.1

A) Cell nuclei were stained with Dapi. B) Indicates a CK_{pos} cell.
C) Alludes a cell with SOX-2_{pos} phenotype. D) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. E) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{pos}, SOX-2_{pos}, $CD34_{neg}$ and/or $CD45_{neg}$, magnification at 63x.

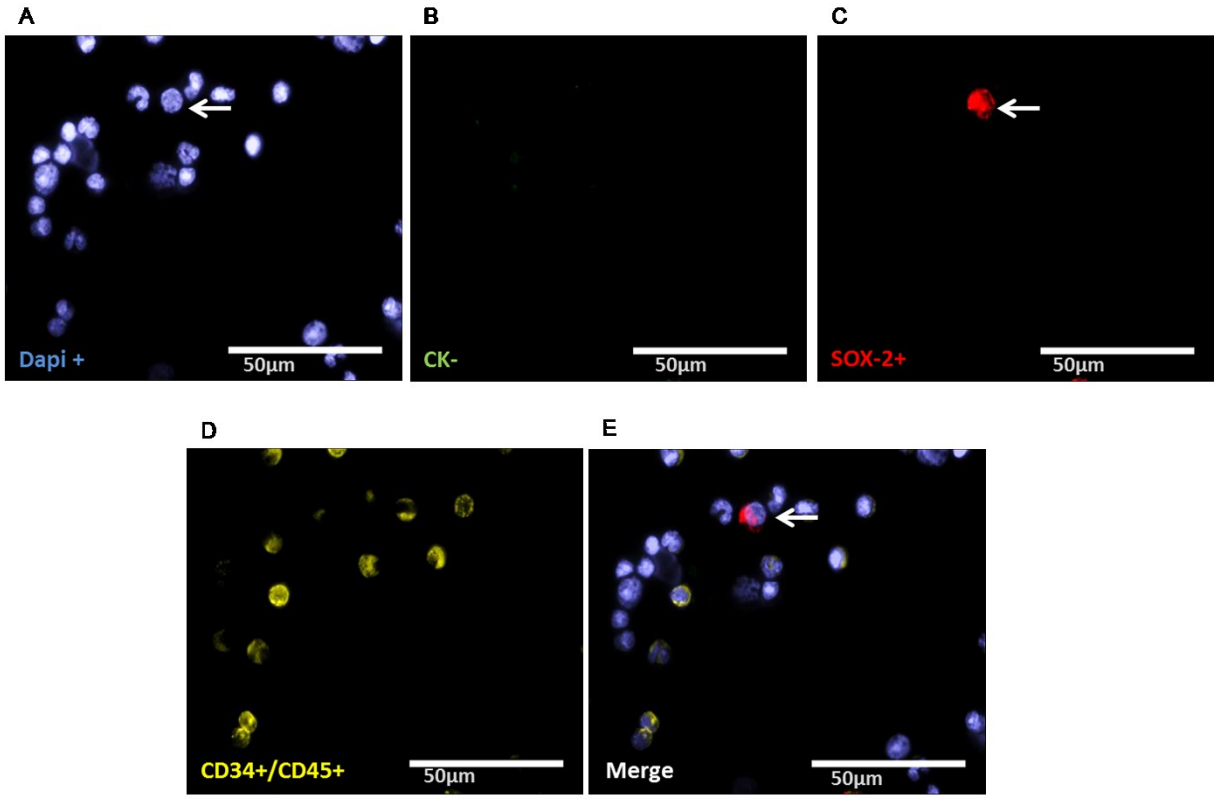
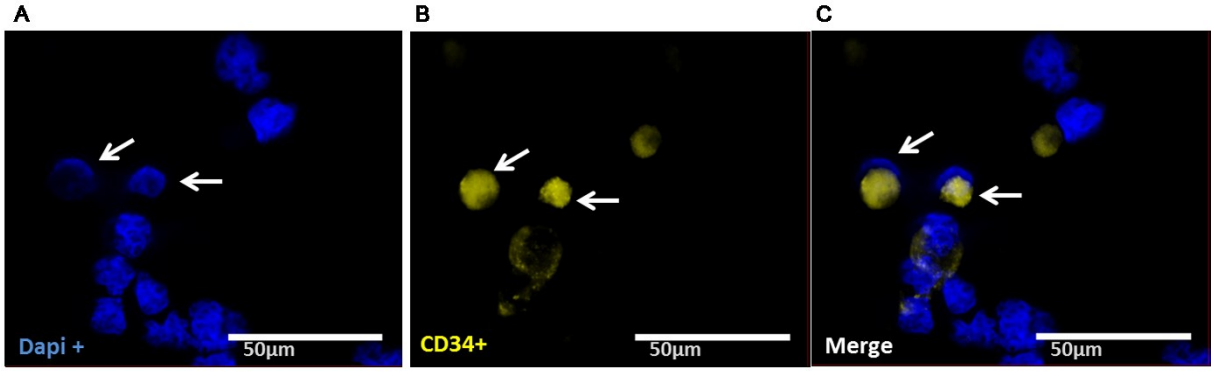


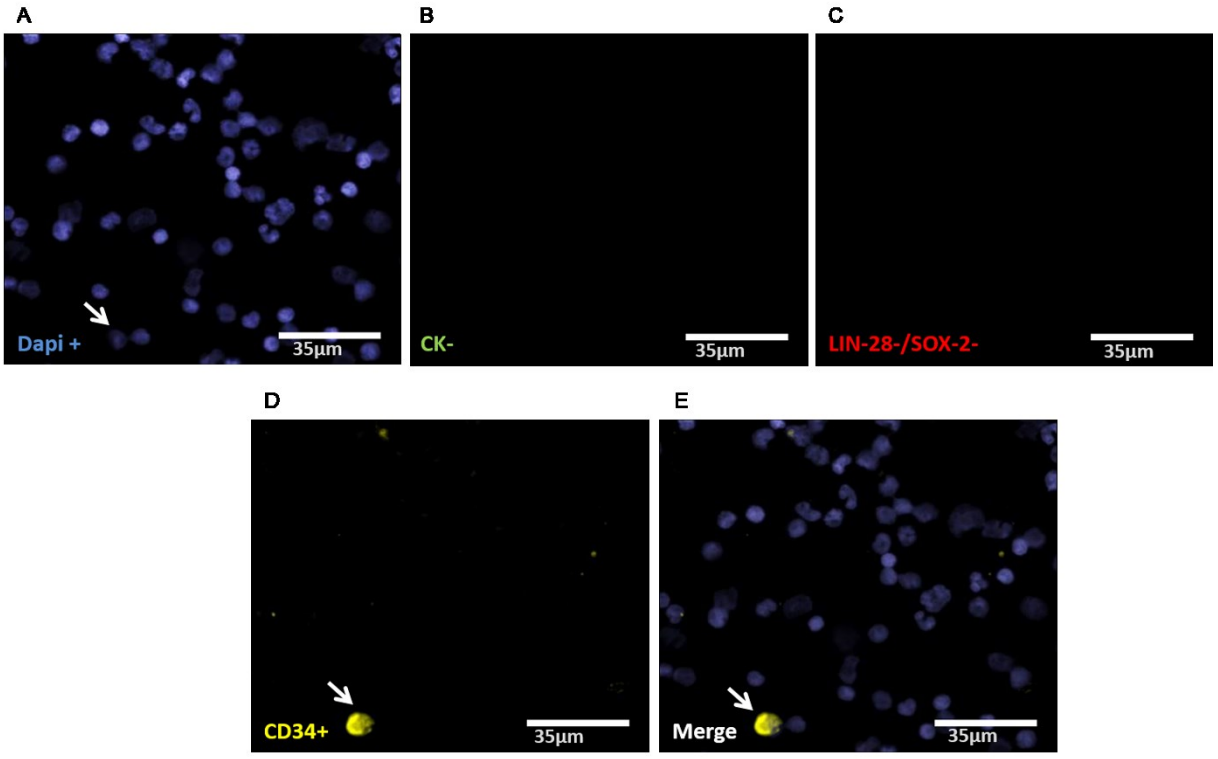
Fig.5. Representative four-fold immunofluorescence staining for CK_{neg}/$SOX\text{-}2_{pos}$ cells after therapy of patient No.1

A) Cell nuclei were stained with Dapi. B) Indicates a CK_{neg} cell. C) Alludes a cell with $SOX\text{-}2_{pos}$ phenotype. D) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. E) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{neg}, $SOX\text{-}2_{pos}$, $CD34_{neg}$ and $CD45_{neg}$, magnification at 63x.



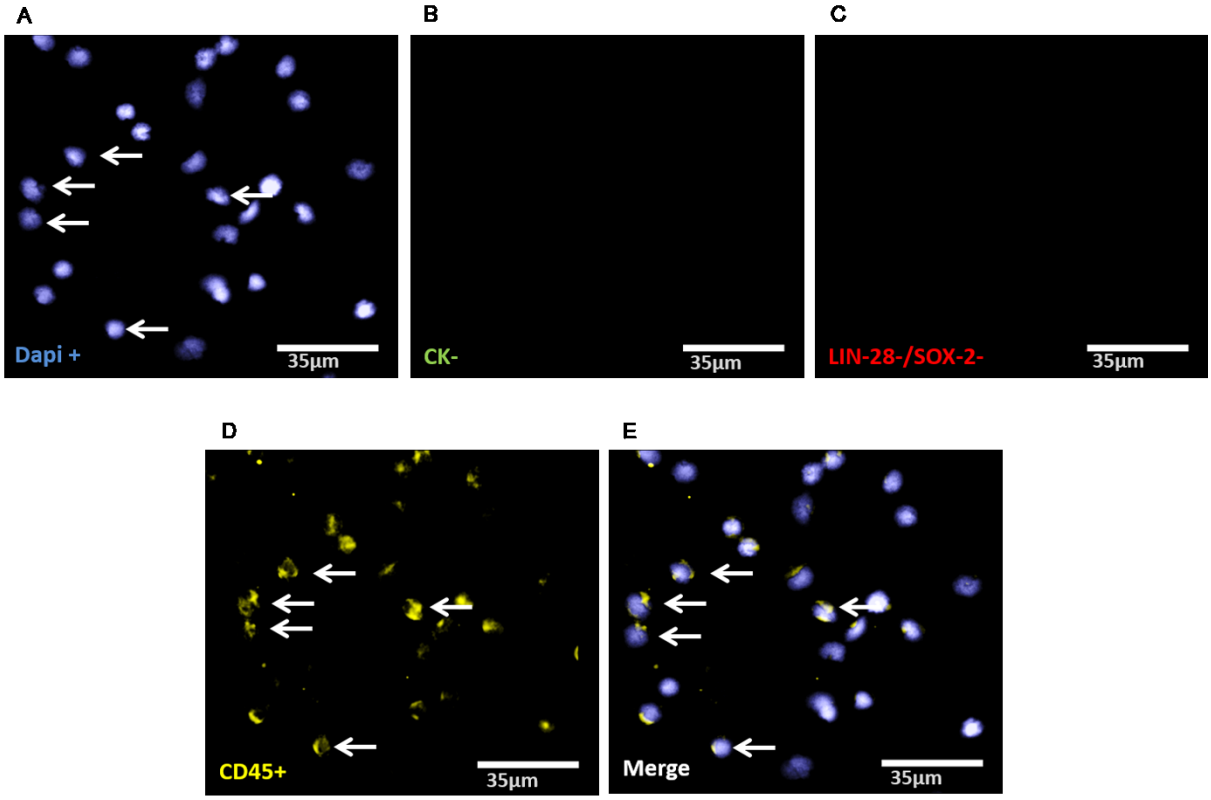
Suppl. Fig.1. Representative staining of Immunofluorescence staining of the Kasumi-1 cell line used as positive control for the detection of hematopoietic stem cells ($CD34_{pos}$).

A) Cell nuclei were stained with Dapi. B) The arrows show two $CD34_{pos}$ cells. C) Indicates a merge of $Dapi_{pos}$ and $CD34_{pos}$ cells, magnification at 63x.



Suppl.Fig.2. Representative four-fold immunofluorescence staining for detection of hematopoietic stem cells ($CD34_{pos}$) in the bone marrow of a healthy donor using primary and secondary antibody for LIN-28 and SOX-2.

A) Cell nuclei were stained with Dapi. B) Indicates CK_{neg} cells. C) Alludes a cell with $LIN\text{-}28_{neg}/SOX\text{-}2_{neg}$ phenotype. D) Shows $CD34_{pos}$ cell. E) Indicates a merge of a hematopoietic stem cell with $Dapi_{pos}$, CK_{neg}, $SOX\text{-}2_{neg}/LIN\text{-}28_{neg}$, $CD34_{pos}$ phenotype, magnification at 40x.



Suppl. Fig.3. Representative four-fold immunofluorescence staining for detection of leukocytes ($CD45_{pos}$) in the bone marrow of a healthy donor using primary and secondary antibody for LIN-28 and SOX2. .

A) Cell nuclei were stained with Dapi.B) Indicates CK_{neg} cells. C) Alludes a cell with $LIN\text{-}28_{neg}/SOX\text{-}2_{neg}$. D) Shows a $CD34_{pos}$ cell. E) Indicates a merge of hematopoietic stem cells with $Dapi_{pos}$, CK_{neg}, $SOX\text{-}2_{neg}/LIN\text{-}28_{neg}$, $CD45_{pos}$ phenotype, magnification at 40x.

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ERCC1-expressing circulating tumor cells as a potential diagnostic tool for monitoring response to platinum-based chemotherapy and for predicting post-therapeutic outcome of ovarian cancer

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ABSTRACT

Background: We recently showed that the presence of ERCC1$^+$CTCs is an independent predictive biomarker for platinum-resistance and poor prognosis of ovarian cancer. The goal of our current research was to determine how the auxiliary assessment of ERCC1-transcripts influences overall CTC-detection rate. We extended this investigation from an initially predictive setting to paired pre- and post-therapeutic blood analysis in order to see, whether ERCC1$^+$CTCs dynamics mirror response to chemotherapy.

Methods: 65 Paired blood samples (10ml) of primary ovarian cancer patients at primary diagnosis and after chemotherapy were studied for CTCs with the AdnaTest *OvarianCancer* (QIAGEN Hannover GmbH). We analyzed the tumor-associated transcripts EpCAM, MUC-1 and CA-125. ERCC1-transcripts were investigated in a separate approach by singleplex RT-PCR.

Results: Auxiliary assessment of ERCC1-transcripts enhanced the overall CTC-detection rate up to 17%. ERCC1$^+$CTCs (defined as positive for one of the AdnaTest markers plus ERCC1-positivity) were detected in 15% of patients at primary diagnosis and in 12% after chemotherapy. The presence of ERCC1$^+$CTCs after chemotherapy correlated with platinum-resistance (P=0.01), reduced PFS (P=0.0293) and OS (P=0.0008) and their persistence indicated poor post-therapeutic outcome (PFS: P=0.005; OS: P=0.0058). Interestingly, the assessment of ERCC1-transcripts alone was sufficient for the detection of prognostic relevant ERCC1-expressing CTCs.

Conclusion: Auxiliary assessment of ERCC1-transcripts expands the phenotypic spectrum of CTC detection and defines an additional overlapping fraction of ERCC1-expressing CTCs, which are potentially selected by platinum-based chemotherapy. Specifically, we suggest that ERCC1$^+$CTCs could additionally be useful as a surrogate for monitoring platinum-based chemotherapy and to assess the post-therapeutic outcome of ovarian cancer.

INTRODUCTION

Epithelial ovarian cancer is the fifth leading cause of cancer death of women in Europe and the United States and the second most common gynecological malignancy [1]. Most cases are diagnosed in advanced stages and, although response rates to chemotherapy reach up to 80%, the majority of patients cannot be cured. Standard treatment of advanced ovarian cancer is primary surgery aiming at complete macroscopic tumor resection followed by platinum- and paclitaxel-based chemotherapy, which has been shown to prolong progression free survival (PFS) as well as overall survival (OS) [2]. Postoperative residual tumor is one of the most important prognostic factors in advanced ovarian cancer [3-5]. However, despite advances in treatment, more than half of all patients will experience recurrence, resulting in poor overall survival [6].

Importantly, resistance to platinum-based chemotherapy, which can be caused by e.g. enhanced DNA-repair capacity of tumor cells, occurs in about 15-20% of patients and constitutes one of the most recognized clinical challenges for ovarian cancer [7]. The nucleotide excision repair (NER) pathway is a key pathway involved in mediating resistance or sensitivity to platinum-based chemotherapeutic agents. The excision repair cross-complementation group 1 (ERCC1) protein plays a key role in NER. It dimerizes with xeroderma pigmentosum complementation group F (known as ERCC4) and mediates the excision of DNA-platinum adducts, typically induced by platinum-based chemotherapy [8]. ERCC1-expression has been extensively studied in primary tumor tissue of several cancer entities, including ovarian cancer, and has been proposed as a potential predictor for response to platinum-based chemotherapy. However, this concept has been controversial, particularly in the context of immunohistochemical ERCC1-detection, and has not been implemented into clinical routine so far [9-20]. Taking into account that primary tumor tissue is typically available only at primary diagnosis, it would be valuable to establish a non-invasive blood-based biomarker for stratifying response to platinum-based chemotherapy at primary diagnosis and for guiding individualized therapy decisions in the future. We recently showed that the presence of ERCC1^{+}CTCs (circulating tumor cells) at primary diagnosis of ovarian cancer, a potentially platinum-resistant CTC-subgroup, is an independent predictive biomarker for primary platinum-resistance and poor prognosis of ovarian cancer [21]. We now explored in more detail, in how far the auxiliary assessment of ERCC1-transcripts influences overall CTC-detection rate and whether

this molecular marker may improve the phenotypic range of CTC-detection by the AdnaTest *OvarianCancer* platform. We essentially extended this investigation from an initially predictive setting to paired pre- and post-therapeutic blood analysis and explored clinical relevance of ERCC1$^+$CTC dynamics in response to platinum-based chemotherapy.

RESULTS

Influence of auxiliary ERCC1-transcript assessment on the CTC-detection rate

We previously have demonstrated that ERCC1 extends clinical information of CTCs as a prognostic biomarker to the prediction of platinum-resistance at primary diagnosis of ovarian cancer [21]. We now explored in more detail how additional assessment of ERCC1 influences the overall detection rate of CTCs in 65 paired pre-operative and post-chemotherapeutic blood samples from ovarian cancer patients. First we assessed the marker transcripts according to the AdnaTest *OvarianCancer* in its previous configuration. CTC-positivity of this assay was indicated by the detection of at least one of the transcripts EpCAM, MUC-1 or CA-125, herein referred to as “AdnaTest$^+$”. Furthermore, we now considered ERCC1-transcripts as an additional marker for CTC-detection.

Figure 1A summarizes the detected CTC-types and shows their relative proportions among the studied ovarian cancer patients. In 8% of patients AdnaTest-positivity was exclusively observed. In 17% we detected exclusively ERCC1-positive CTCs and in 15% we observed dual positivity for the AdnaTest and ERCC1. Subsequently, we were interested in how auxiliary assessment of ERCC1 influences the overall detection rate of CTCs in ovarian cancer. Therefore, we compared overall CTC-detection rates across several defining criteria for “CTC-positivity”, with the presence of ERCC1-transcripts as an additional alternative criterion or as obligatory requirement, respectively (Figure 1B): We observed a CTC-detection rate of 23% before surgery, comprised of patients with only AdnaTest positivity and dual AdnaTest/ERCC1-positive patients. Detection rates were substantially increased up to 40% if ERCC1 was considered as a further alternative marker for CTC-positivity (AdnaTest$^+$ OR ERCC1$^+$). Since this CTC-definition now comprises a further subgroup of patients with exclusively ERCC1-expressing CTCs. This subgroup alone can be detected with an overall detection rate of 17% (AdnaTest$^-$ AND ERCC1$^+$). Lastly, according to a more stringent definition of combined positivity (AdnaTest$^+$ AND ERCC1$^+$), overall detection

decreased to 15%.

After platinum-based chemotherapy the proportion of CTC-subtypes and their overall detection rates among the above mentioned CTC-definition criteria were grossly comparable with those found before therapy (Figure 1C+D).

ERCC1$^+$CTCs predict post therapeutic outcome

The median follow up time for PFS was 37 months (range 4-120 months) resulting in 36 (55%) relapses while 28 patients (43%) presented with no relapse. After a median follow-up time of 45 months (range 11-117 months) for OS, 42 patients (65%) were still alive and 23 patients (35%) had died (Table 1).
We first explored the clinical relevance of ERCC1-expressing CTCs in post-therapeutic blood samples. For this purpose, in accordance with our previous publication [21], we primarily focused on the most stringent definition of ERCC1-expressing CTCs, which is based on the previous AdnaTest markers (EpCAM, MUC-1, CA-125) and encludes ERCC1-positivity as an additional obligatory requirement (AdnaTest$^+$ AND ERCC1$^+$). This cell population is from now on referred to as ERCC1$^+$CTCs. The presence of post-therapeutic ERCC1$^+$CTCs significantly correlated with decreased PFS (p=0.0293) and OS (p=0.0008, Figure 2A+B). Furthermore, the presence of ERCC1$^+$CTCs after chemotherapy correlated with primary platinum-resistance (p=0.01, data not shown).

ERCC1$^+$CTC dynamics mirror response to platinum-based chemotherapy

We were further interested in how the levels of ERCC1$^+$CTCs in our patients changed in response to platinum-based chemotherapy. A stratification of our study patients according to “ERCC1$^+$CTCs dynamic subgroups” is presented in Figure 3. The majority of patients were negative for ERCC1$^+$CTCs throughout (77%, “neg-neg”) treatment. In 11% of patients we observed ERCC1$^+$CTCs before surgery, which disappeared after platinum-based chemotherapy (“pos-neg”). Moreover, 8% of patients were initially negative and ERCC1$^+$CTCs newly appeared after therapy (“neg-pos”). Finally, in 5% of patients, persistent ERCC1$^+$CTCs were observed before surgery and after chemotherapy (“pos-pos”). Interestingly, patients with persistent positivity for ERCC1$^+$CTCs before surgery and after chemotherapy (ERCC1$^+$CTC “pos-pos”) had a very poor PFS (p=0.0053) and OS (p=0.0058, Figure 4 A+B) compared to all other dynamic subgroups together (“neg-neg” or “pos-neg” and “neg-pos”). Furthermore, we observed the trend that patients with newly acquired ERCC1$^+$CTCs after

chemotherapy (“neg-pos”) had a shorter PFS by trend (p=0.2871) and a significantly shorter OS (p=0.0202) than the “neg-neg” group and the “pos-neg” group together (Figure 4 C+D).

The assessment of ERCC1-transcripts alone is a surrogate for the detection of prognostically relevant CTCs

We were interested in how prognostic information as described above was retained when ERCC1-transcripts alone were assessed. Therefore, we exclusively focused on ERCC1-transcript expression, irrespectively of EpCAM, MUC-1 or CA-125 positivity and re-performed survival analysis. The presence of post-therapeutic ERCC1-transcript positivity alone indicated reduced PFS (p=0.0158) and OS (p=0.0377, Figure 5 A+B). Once more, a stratification of our study patients according to “ERCC1 dynamic subgroups” was performed and is presented in Supplementary Figure 1. The majority of patients were negative for ERCC1-transcritps throughout treatment (57%, “neg-neg”). In 12% of patients we observed ERCC1-positivity before surgery which disappeared after platinum-based chemotherapy (“pos-neg”). Moreover, 11% of patients were initially negative and ERCC1^{+}CTCs newly appeared after therapy (“neg-pos”). Finally, in 20% of patients, persistent ERCC1^{+}CTCs were observed before surgery and after chemotherapy (“pos-pos”). More interestingly, as already reported for ERCC1^{+}CTCs, the ERCC1 “pos-pos” subgroup also had a significantly decreased PFS (p=0.0021) and OS (p=0.0327), compared to all other dynamic subgroups together (Figure 5 C+D). Of note is that the statistical significance level of these findings was generally lower compared to the prognostic relevance of ERCC1^{+}CTCs, which referred to combined CTC- and ERCC1-positivity.

The number of patients at risk in each of the subgroups shown in Figures 2, 4 and 5 are documented in Supplementary Table 1.

DISCUSSION

In the present study we demonstrate that the additional assessment of ERCC1-transcripts enhanced overall CTC detection rate in ovarian cancer patients. It also defines an additional overlapping fraction of ERCC1-expressing CTCs, which are potentially selected by platinum-based chemotherapy. We also describe that the assessment of CTC-derived ERCC1-transcripts alone was almost equivalently sufficient in order to detect ERCC1-expressing prognostic relevant CTCs. We further showed that the presence of ERCC1$^+$CTCs after chemotherapy correlated with post-therapeutic outcome of ovarian cancer and, particularly, dynamics of ERCC1$^+$CTCs mirrored response to platinum-based chemotherapy.

We have already demonstrated that a) additional detection of ERCC1-transcripts extented clinical value of CTCs from a prognostic biomarker to an independent predictor of platinum-resistance at primary diagnosis of ovarian cancer and b) ERCC1$^+$CTCs may constitute a distinct subgroup of CTCs with a potentially platinum-resistant phenotype [21]. We now confirmed that auxiliary assessment of ERCC1-transcripts considerably expanded the phenotypic spectrum of CTC-detection in pre- and post-therapeutic blood samples and that ERCC1 obviously marks a definable CTC-phenotype with overlap to the CTC-population, as detected by the AdnaTest *OvarianCancer*. Therefore, in a considerable number of patients, CTC-derived ERCC1-expression was accompanied by co-expression of at least one of the standard markers for CTC-detection (EpCAM or MUC-1 or CA-125). Given the experimental setting of the AdnaTest, we cannot distinguish whether this co-expression was derived from CTCs actually co-expressing these markers on the same cell, or from separate CTC-populations which were concomitantly present in the “pool” of immunomagnetically enriched CTCs from a given blood sample. However, we also observed a minor subset of patients who were exclusively positive for ERCC1-transcripts. We suppose that these patients harbor epithelial-associated CTCs in their blood, which express EpCAM or MUC-1 antigens on their surface. These CTCs were captured by the AdnaTest selection procedure which targets EpCAM and MUC-1 surface epitopes. However EpCAM and MUC-1 transcripts seemed to be downregulated on the transcriptional level in these isolated CTCs. Discordances between protein and transcript expression profiles of a cell could be due to post-transcriptional modifications of messenger RNA or differences in the half-life time between messenger RNA and their corresponding proteins [22-24].

The broad heterogeneity of CTCs in the blood of cancer patients, including ovarian cancer, has already been indicated by several independent reports [25-27]. We may hypothesize that ERCC1-expressing CTCs play a dominant role during the course of the disease, which is corroborated by the fact that the rate of exclusively ERCC1-positive CTCs did not decrease after platinum-based chemotherapy. We have already shown that breast cancer patients with CTCs detected after neoadjuvant chemotherapy were associated with tumor stem cell characteristics and ERCC1-expression [28]. This may suggest a potential selection of this CTC-subset by chemotherapy. However, due to the fact that exclusively ERCC1-expressing CTCs rarely occurred in our patient cohort, we were not able to analyze prognostic significance of this particularly interesting CTC-subset in a statistically substantiated manner.

Independent of ERCC1 assessment, we previously confirmed the negative prognostic impact of CTCs before surgery and after chemotherapy, as detected by the AdnaTest *OvarianCancer* [21, 29]. Nevertheless, our recent and current results strongly support our perception that the auxiliary assessment of ERCC1-transcripts provides complementary clinical information. In addition, the auxiliary assessment of ERCC1-transcripts after chemotherapy alone, as well as their expression dynamics in pre- and post-therapeutic blood samples, was almost equivalently sufficient as surrogate for a CTC-population. This might be useful for predicting post-therapeutic outcome and for monitoring platinum-based chemotherapy. Given the additional strong prognostic impact of the standard AdnaTest marker transcripts MUC-1, EpCAM and CA-125 [21, 29] and considering that the statistical significance level slightly declined when only ERCC1-transcripts were assessed, a combined condition, which assumes ERCC1-positivity in addition to the detection of at least one of the AdnaTest markers (referred to as $ERCC1^{+}$CTCs in our study), appears to be most favorable in terms of a blood-based prognostic biomarker.

So far, any functional characteristics of ERCC1-expressing CTCs in the blood of ovarian cancer patients are unknown. Since our study was performed exclusively from a “biomarker perspective”, we can only assume that ERCC1-(over)expressing CTCs in the blood may be characterized by an enhanced, preexisting or newly acquired capacity to resolve DNA-platinum-adducts, consequently bypassing cisplatin-mediated cytotoxicity and possibly converting to a molecular phenotype of “on-target” platinum-resistance [8]. This assumption is further supported by a recent investigation which directly analyzed the presence of DNA-platinum adducts in single CTCs of advanced

non-small cell lung cancer (NSCLC) patients. In this context, it was suggested that the kinetics of these adducts in pre- and post- therapeutic blood samples could be a potential biomarker for response prediction and dose individualization of platinum-based chemotherapy [30]. Consecutively, ERCC1^{+}CTCs may survive multiple cycles of chemotherapy and, in line with the fact that metastasis-initiating cells can be present among CTCs in the blood [31], persistent ERCC1^{+}CTCs with a platinum-resistant phenotype could have the potential to initiate recurrence, resulting in poor clinical outcome. Taking into consideration that ERCC1^{+}CTCs are strong prognostic factors in the post-therapeutic situation, particularly in case of persistent positivity, our data may also indicate that platinum-resistant ERCC1^{+}CTCs could be directly selected upon platinum-based chemotherapy. Further functional studies will be necessary in order to prove this hypothesis. Another interesting question for future studies will be, how CTC-derived ERCC1-expression is related to EMT- and stem-like characteristics of CTCs. In this regard, we recently demonstrated that the negative prognostic impact of the presence and/or persistence of disseminated tumor cells in the bone marrow of ovarian cancer patients after platinum based chemotherapy was associated with stem cell character [32].

Conclusively, due to the limited number of patients, our study is explorative and hypothesis generating. Nevertheless, ERCC1 marks a subpopulation of CTCs which might be useful for monitoring platinum-based chemotherapy and for assessing post-therapeutic outcome of ovarian cancer patients. We provide rationale to validate clinical utility of ERCC1^{+}CTCs among large multicenter clinical trials and to further elucidate their functional and tumor biological significance. Alternatively, patients with ERCC1*CTCs may profit from an early initiated and dose-intense maintenance therapy with e.g. Bevacizumab or PARP-inhibitors. Furthermore, this high risk patient group might be amenable to platinum-sensitizing therapies in the future, which are increasingly proposed in preclinical studies [33-35] and already ongoing clinical trials (NCT01164995). Using CTCs as liquid biopsy tool for individual therapy optimization, a multi-marker gene panel, compromising all CTC subgroups, will be useful to monitor patients during the course of the disease [36].

PATIENTS AND METHODS

Patient characteristics

The present study was conducted at the Department of Gynecology and Obstetrics at the University Hospital of Essen, Germany. A total of 65 patients diagnosed between 2006 and 2014 with histologically confirmed epithelial ovarian cancer were analyzed. Clinical characteristics of the patients are documented in Table 1. Informed written consent was obtained from all patients and the study was approved by the Local Ethics Committee (05-2870) and performed according to the declaration of Helsinki. Tumors were classified according to the WHO classification of tumors of the female genital tract. Grading was conducted using the grading system proposed by Silverberg [37] and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique [38]. The whole study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping were performed. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if macroscopic complete tumor resection was achieved intraperitoneally following actual guidelines. All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m^2. Tumors were clinically defined as platinum-resistant if they recurred within six months after the completion of platinum-based chemotherapy.

Enrichment and Molecular Characterization of CTCs

Peripheral blood (2x5 ml) from each patient was collected in EDTA tubes (Sarstedt & Co.) and processed within 4h for the enrichment of CTCs and subsequent expression analysis according to Adnatest *OvarianCancer* (QIAGEN, Hannover GmbH, Langenhagen, Germany). The test has been described in detail [21]. Briefly, CTCs were immunomagnetically selected using the AdnaTest *OvarianCancerSelect* targeting epithelial cell adhesion molecule EpCAM (also known as GA733-2), Mucin-1, cell surface associated (MUC-1) and cell surface associated Mucin-16 (also known as CA-125). Subsequently, RNA was isolated and gene expression analysis was performed by reverse-transcription (RT) and multiplex RT-PCR detecting EpCAM, MUC-1, and CA-125 (AdnaTest *OvarianCancerDetect)*. ERCC1-transcripts were investigated in a separate approach by singleplex RT-PCR. β-actin served as an internal control and PCR-products were quantified on the Agilent Bioanalyzer as follows: Blood samples of 20 healthy donors and healthy donor blood samples spiked

with two or five cells IGROV1 were analyzed using the AdnaTest *OvarianCancer Select/Detect* for overexpression of EpCAM, MUC-1 and CA-125. The resulting PCR fragments were analyzed with the Agilent Bioanalyzer and the resulting data were checked for sensitivity and specificity to be ≥ 90% applying a 0.15 ng/µl fragment concentration as a cut of value for each of the markers. For the required specificity of >90%, as defined in the test performance criteria, the resulting cut off value was defined as 0.15 ng/µl fragment concentration. At a cut-off value of 0.15 ng/µl specificity is 95% and the corresponding recovery rate is 80% for two cells and 100% for five cells, respectively.

ERCC1-positivity was defined by an amplicon concentration >0.2ng/µl. Sensitivity and specificity were evaluated in 20 healthy donors and 99 patients with primary ovarian cancer using ROC analysis. At a cut-off value of 0.17 ng/µl, 95% specificity is reached and the corresponding clinical sensitivity is 46.5% (Supplementary Figure 2).

Amplicons with the following sizes were generated: EpCAM: 396bp; MUC-1: 293bp; CA-125: 432bp; ERCC1: 366bp; and β-actin: 114bp.

Statistical Analysis

Survival curve plots and Hazard Ratio calculations were done using SAS (9.4). Survival intervals were screened from the time of CTC detection at first diagnosis to the time of clinical event (either death or first time of relapse) or last contact. Kaplan-Meier curves were assessed using the log-rank test to evaluate univariate significance of the binary grouping parameters. Fisher exact tests were performed to confirm significance. Survival curve plots and Hazard Ratio calculations were done using SAS (9.4).

Tables

Tab.1 Patient characteristics at the time of primary diagnosis

Total	**65**
Age	median 61 years, (27-92)
FIGO stage	
I-II	11 (17%)
III	41 (63%)
IV	13 (20%)
Nodal status	
N_o	24 (37%)
N_1	28 (43%)
N_x	13 (20%)
Grading	
I-II	28 (43%)
III	37 (57%)
Unknown	0 (0%)
Residual tumor	
Macroscopic	
Complete resection	38 (58%)
Any residual tumor	27 (42%)
Histologic type	
Serous	52 (80%)
Mucinous	9 (14%)
Other	4 (6%)
Survival	
PFS [1]	median 37 months, (4-120 months)
OS[2]	median 45 months, (11-117 months)
Alive	42 (65%)
Dead	23 (35%)
Unknown	0 (0%)
Recurrence	
No relapse	28 (43%)
Relapse	36 (55%)
Unknown	1 (2%)

[1]PFS: progression-free survival, [2]OS: overall survival

FIGURE LEGENDS

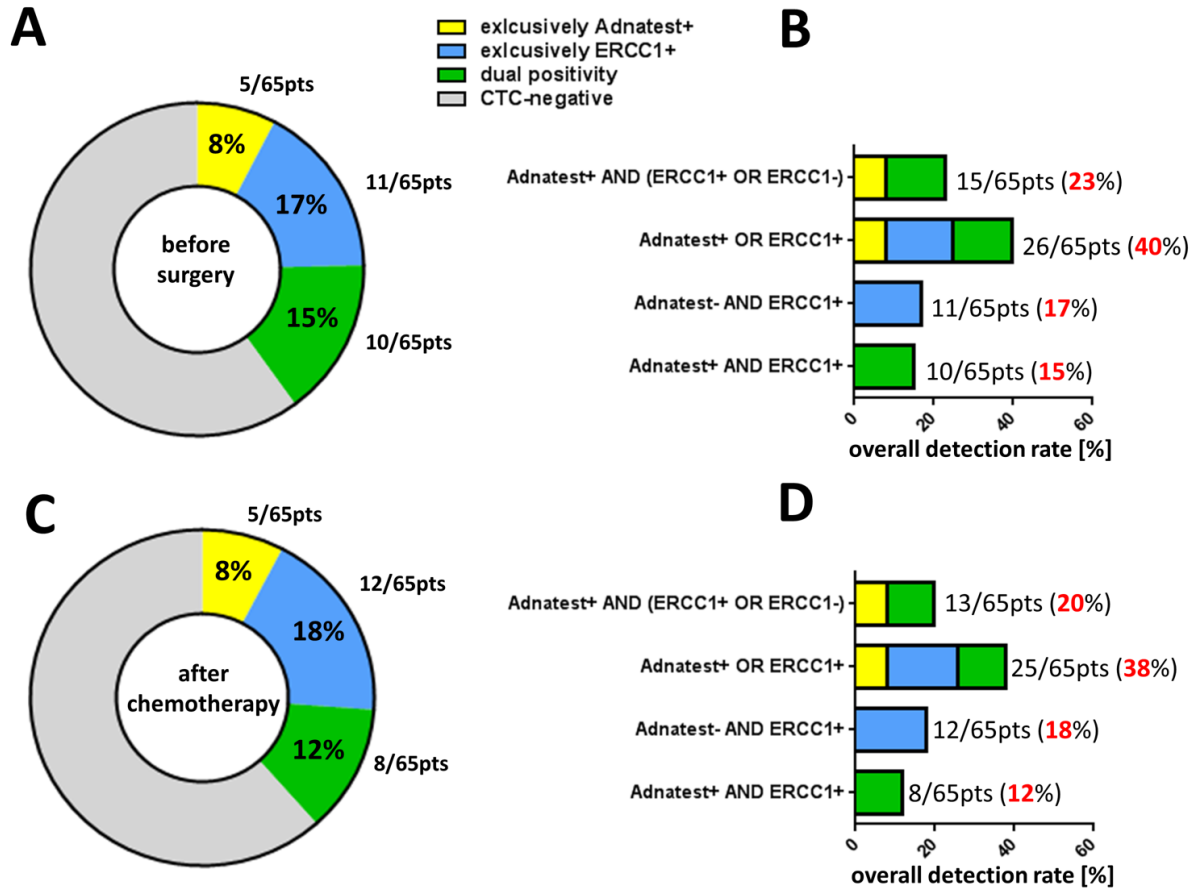


Fig.1: Influence of auxiliary ERCC1-transcript assessment on CTC-detection rate

A The pie chart shows the different CTC-types and their relative proportions among the studied ovarian cancer patients before surgery (n=65). Percentages indicate the proportion of patients with exclusively-Adnatest-positivity (yellow), exclusively-ERCC1-positivity (blue), dual-positivity for Adnatest/ERCC1 (green) and CTC-negative patients (grey). B The stacked bar chart summarizes four CTC-definition criteria, considering ERCC1 as additional transcript marker and shows, how this is translated into different overall CTC-detection rates. C+D These illustrations depict the same type of analysis as reported above, however refer to paired blood samples analyzed after platinum-based chemotherapy (n=65). In all figures, absolute patient numbers in each subgroup are indicated.

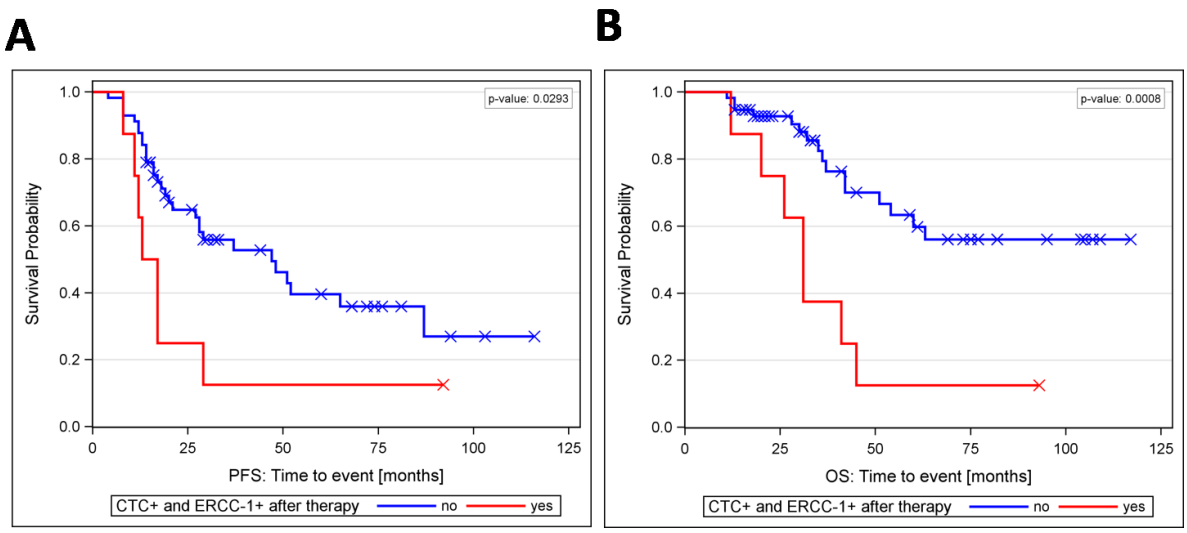


Fig.2: Prognostic relevance of ERCC1⁺CTCs after chemotherapy

A patient was considered positive for ERCC1⁺CTCs if at least one of the AdnaTest transcript markers (EpCAM, MUC-1 or CA-125) was detected, in addition to ERCC1-positivity. The Kaplan-Meier analysis shows (**A**) progression-free survival and (**B**) overall survival of patients with detectable ERCC1⁺CTCs after platinum-based chemotherapy (bottom curves) in comparison to patients with non-detectable ERCC1⁺CTCs (top curves).

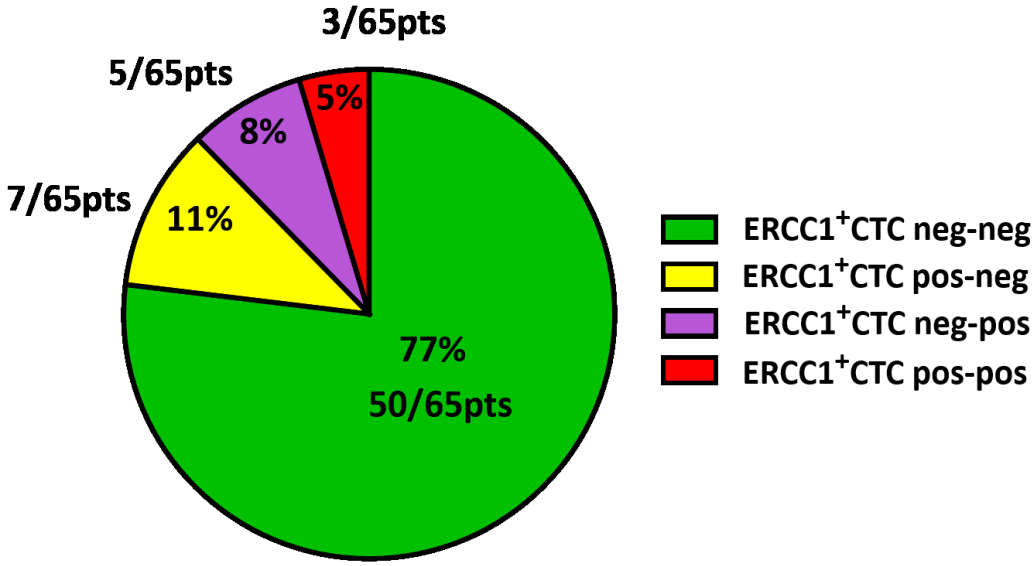


Fig. 3: Dynamics of ERCC1⁺CTCs in the course of platinum-based chemotherapy

A patient was considered positive for ERCC1⁺CTCs if at least one of the Adnatest transcript markers (EpCAM, MUC-1 or CA-125) was detected, in addition to ERCC1-positivity. The pie chart shows a stratification of the study cohort (n=65) into different subgroups, according to the dynamics of ERCC1⁺CTCs before surgery and after chemotherapy. Besides the group of patients, who were negative for ERCC1⁺CTCs throughout (ERCC1⁺CTCs **neg-neg**), we observed patients, who became negative after chemotherapy (ERCC1⁺CTCs **pos-neg**), patients with newly acquired positivity after chemotherapy (ERCC1⁺CTCs **neg-pos**) or persistently positive patients (ERCC1⁺CTCs **pos-pos**). Percentages and absolute patient numbers are indicated.

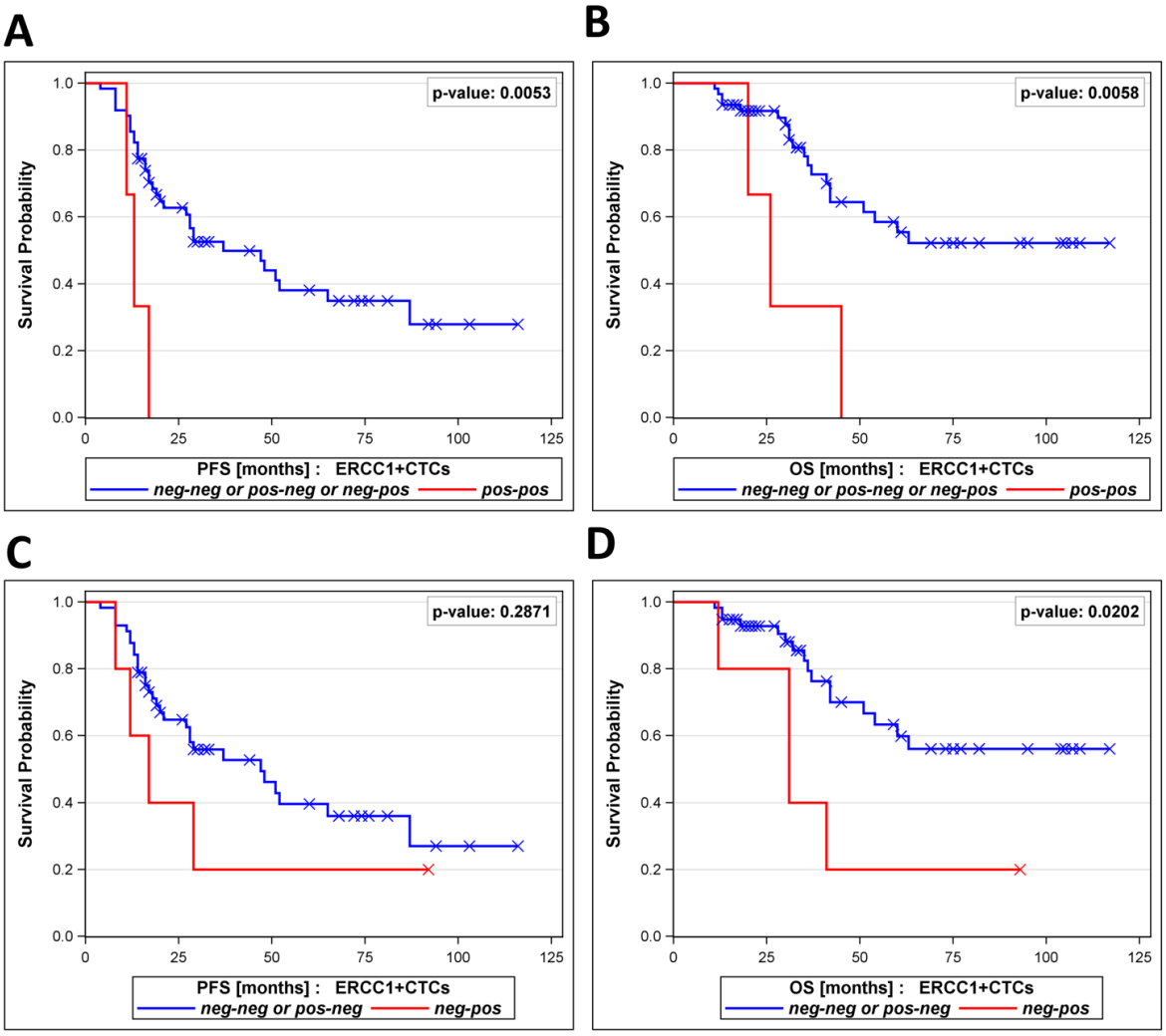


Fig. 4: Prognostic relevance of persistent ERCC1⁺CTCs

The Kaplan-Meier plots show (**A**) progression-free survival and (**B**) overall survival of patients with persistent positivity for ERCC1⁺CTCs in their blood (ERCC1⁺CTC **pos-pos**, bottom curves), in comparison to all other dynamic subgroups together (ERCC1⁺CTC **pos-neg** / **neg-pos** / **neg-neg,** top curves). Moreover, Kaplan-Meier plots show (**C)** progression-free survival and (**D**) overall survival of patients with newly acquired positivity for ERCC1⁺CTCs (ERCC1+CTC **neg-pos**, bottom curves), in comparison to the dynamic subgroups ERCC1⁺CTC **pos-neg** and ERCC1⁺CTC **neg-neg**, together (top curves).

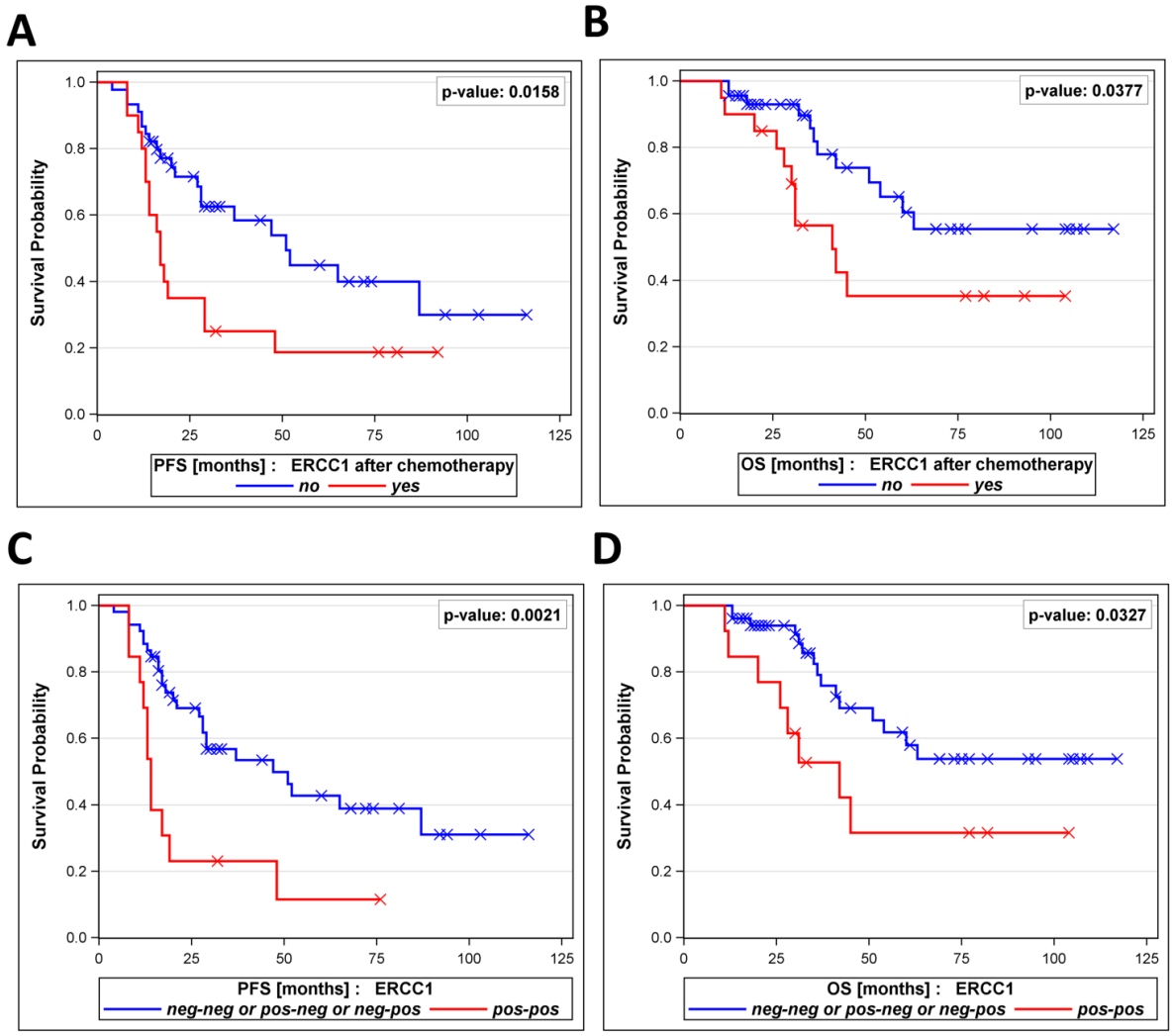
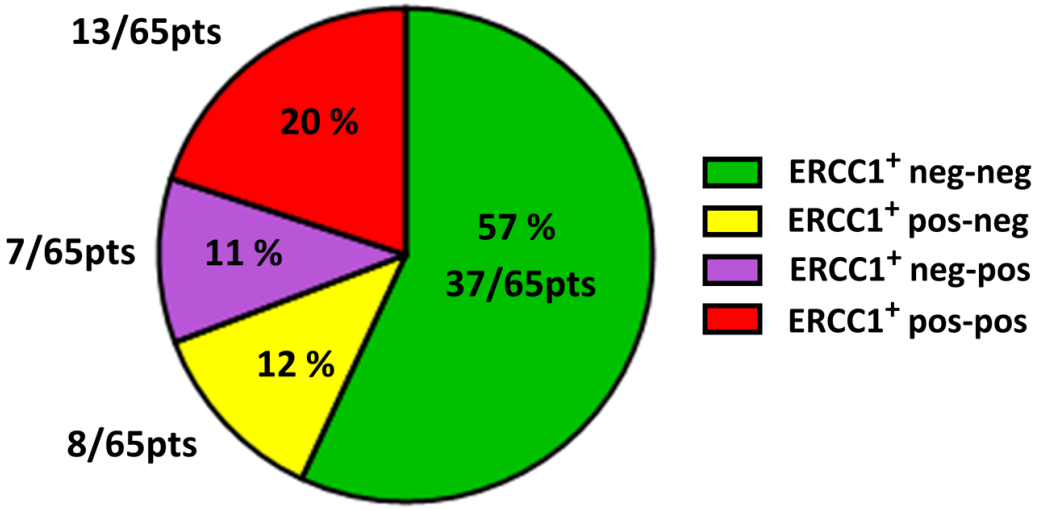


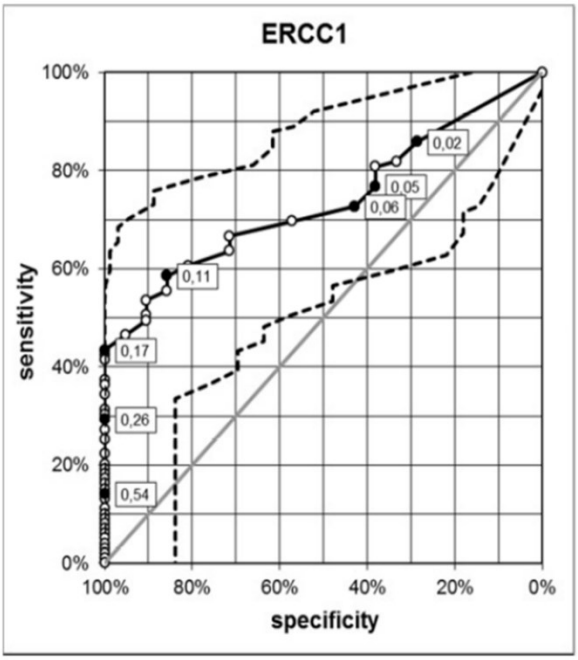
Fig.5: Prognostic relevance of ERCC1-transcripts alone

This analysis refers to the prognostic relevance of ERCC1-transcripts alone, irrespectively of the Adnatest transcript markers EpCAM, MUC-1 or CA-125. The Kaplan-Meier plots show (**A**) progression-free survival and (**B**) overall survival of patients with ERCC1-positivity after platinum-based chemotherapy (bottom curves) in comparison to patients with non-detectable ERCC1-transcripts (top curves). Moreover, Kaplan-Meier plots show (**C)** progression-free survival and (**D**) overall survival of patients with persistent positivity for ERCC1-transcripts (ERCC1^{+} **pos-pos**, bottom curves) in comparison to all other dynamic subgroups together (ERCC1^{+} **pos-neg** / **neg-pos** / **neg-neg,** top curves).



Suppl. Fig.1: Dynamics of ERCC1-positivity in the course of platinum-based chemotherapy

This analysis refers to the prognostic relevance of ERCC1-transcripts alone, irrespectively of the Adnatest transcript markers EpCAM, MUC-1 or CA-125. The pie chart shows a stratification of the study cohort (n=65) into different subgroups, according to the dynamics of ERCC1-positivity (ERCC1$^+$) before surgery and after chemotherapy. Beside the group of patients, who were negative for ERCC1 throughout treatment (ERCC1$^+$ **neg-neg**), we observed patients, who became negative after chemotherapy (ERCC1$^+$ **pos-neg**), patients with newly acquires positivity after chemotherapy (ERCC1$^+$ **neg-pos**) or persistently positive patients (ERCC1$^+$ **pos-pos**). Percentages and absolute patient numbers are indicated.



N (normal)	N (abnormal)
21	99
ROC CURVE	P1
AUC	0,7367
SE	0,0528
low. CI (0,95)	0,6483
upp. CI (0,95)	0,8130
SPEC	SENS
95%	46,5%
90%	53,5%
80%	60,6%
SENS	SPEC
95%	0,0%
90%	0,0%
80%	38,1%

Suppl. Fig.2: ROC analysis for the determination of ERCC1 sensitivity and specificity.

Blood samples of 20 healthy donors and 99 patients with primary ovarian cancer were analyzed for CTCs with the AdnaTest *OvarianCancer* and for ERCC1 expression applying densitometric fagment quantification using the Agilent Bioanalyzer 2100. The resulting data were checked for sensitivity and specifity. A cut off value was determeined using a ROC analysis. At a cut-off value of 0.17 ng/µl 95% specificity was reached and the corresponding clinical sensitivity was 46.5%. A slightly higher cut-off at 0.2 ng/µl was, however, chosen as kind of a security measure.

Suppl. Tab.1: Univariate analysis of different CTC-subgroups and patient numbers at risk

	at risk (%)					p-value (log rank)
1. PFS	**0**	**25**	**50**	**75**	**100**	
ERCC-1 after chemotherapy						**0.0158**
No	45	25	12	4	2	
yes	20	7	3	3	0	
ERCC-1						
pos-pos	13	3	1	1	0	**0.0021**
neg-neg or pos-neg or neg-pos	52	29	14	6	2	
ERCC-1$^+$CTCs after chemotherapy						**0.0293**
No	57	30	14	6	2	
yes	8	2	1	1	0	
ERCC-1$^+$CTCs						
pos-pos	3	0	0	0	0	**0.0053**
neg-neg or pos-neg or neg-pos	62	32	15	7	2	
ERCC-1$^+$CTCs						
neg-pos	5	2	1	1	0	0.2871
neg-neg or pos-neg	57	30	14	6	2	
	at risk (%)					p-value (log rank)
2. OS	**0**	**25**	**50**	**75**	**100**	
ERCC-1 after chemotherapy						**0.0377**
No	45	31	17	8	5	
yes	20	16	5	5	1	
ERCC-1						
pos-pos	13	10	3	3	1	**0.0327**
neg-neg or pos-neg or neg-pos	52	37	19	10	5	
ERCC-1$^+$CTCs after chemotherapy						**0.0008**
No	57	41	21	12	6	
Yes	8	6	1	1	0	
ERCC-1$^+$CTCs						
pos-pos	3	2	0	0	0	**0.0058**
neg-neg or pos-neg or neg-pos	62	45	22	13	6	
ERCC-1$^+$CTCs						
neg-pos	5	4	1	1	0	**0.0202**
neg-neg or pos-neg	57	41	21	12	6	

The table summarizes univariate analysis according to the Kaplan-Meier curves (Figure 2, 4, 5). The absolute number of patients in each subgroup at a risk of 0%, 25%, 50%, 75% and 100% is indicated for each case.

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EMT-like circulating tumor cells in ovarian cancer patients are enriched by platinum-based chemotherapy

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Key words: ovarian cancer, circulating tumor cells, epithelial-to-mesenchymal-transition, PI3Kα, Akt-2, Twist

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ABSTRACT

Background: Epithelial-to-mesenchymal-transition (EMT) contributes to therapy resistance of ovarian cancer and can particularly be triggered by chemotherapy. However, in how far EMT extends to circulating tumor cells (CTCs) of ovarian cancer patients, is unknown. Therefore, assuming that dissemination of ovarian cancer cells requires at least a partial shift to a mesenchymal phenotype, we analyzed the incidence of EMT-like CTCs in ovarian cancer patients and inquired, how their molecular phenotypes respond to platinum-based chemotherapy.

Methods: Blood samples of ovarian cancer patients at primary diagnosis (n=91) and matched samples after adjuvant chemotherapy (n=31) were evaluated for CTCs with the AdnaTest *OvarianCancer* and *EMT1/StemCell*, analyzing the epithelial-associated transcripts EpCAM, Muc-1 and CA125 and the EMT-associated transcripts PI3Kα, Akt-2 and Twist.

Results: At primary diagnosis of ovarian cancer, epithelial CTCs were detected with an overall incidence of 18%. EMT-like CTCs were more frequently observed (30%) and were mutually exclusive to epithelial CTCs in the majority of patients (82%). In response to platinum-based chemotherapy, we observed an increase in EMT-like CTCs up to 52%, which was accompanied by the “de novo” emergence of dual PI3Kα+Twist positive, EMT-like CTCs.

Conclusion: This is the first explorative study on ovarian cancer, reporting that platinum-based chemotherapy selects for ETM-like CTCs and provokes a shift of molecular phenotypes towards PI3Kα and Twist expressing CTCs, which may reflect clonal tumor evolution. Future investigation will have to determine, whether this subgroup of CTCs may be suitable as therapeutic target or as biomarker for high risk minimal residual disease in ovarian cancer.

Key words: ovarian cancer, circulating tumor cells, epithelial-to-mesenchymal-transition, PI3Kα, Akt-2, Twist

INTRODUCTION

Epithelial ovarian cancer is the fifth leading cause of cancer death among women in Europe and the United States and the second most common gynecological malignancy [1]. Most cases are diagnosed in advanced stages and although response rates to chemotherapy reach up to 80%, the majority of patients cannot be cured. Standard treatment of advanced ovarian cancer is primary surgery aiming at complete resection followed by platinum and paclitaxel based chemotherapy, which has been shown to prolong progression free survival (PFS) as well as overall survival (OS) [2]. Postoperative residual tumor is one of the most important prognostic factors in advanced ovarian cancer [3-5]. Meanwhile, although new multimodal therapeutic concepts, such as antiangiogenic therapy (e.g. Bevacizumab) or PARP-inhibition (only for recurrent patients), have been designed, more than half of all patients still experience recurrence, resulting in a poor overall prognosis [6-8]. Thus, the identification of innovative therapeutic targets and the identification of predicitive and prognostic biomarker concepts are highly desirable.

In this regard, circulating tumor cells (CTCs) in the blood and disseminated tumor cells (DTCs) in the bone marrow (BM) have already been shown to be promising candidates [9]. Despite the fact that CTCs indicate poor prognosis [10-14], we recently reported that excision repair cross-complementation group 1 protein (ERCC-1)-positive CTCs are present in 8% of patients and constitute an independent predictor, not only for OS but also for PFS. Most interestingly, we discovered the presence of ERCC1^{+}CTC at primary diagnosis to be an independent predictor for platinum-resistance, whereas ERCC1-expression in corresponding primary tumor tissue predicted neither platinum-resistance nor prognosis [15]. Moreover, we reported that the presence of DTCs in the BM, as well as their persistence after platinum based chemotherapy, correlates with poor prognosis and is accompanied by stem cell characteristics of DTCs [16].

The broad heterogeneity of CTCs of cancer patients, including ovarian cancer, has already been demonstrated [17-19] and we may speculate that, besides the presence of epithelial, stem cell-like or potentially platinum-resistant ERCC1-expressing CTCs, some other CTC-phenotypes may play a dominant role for therapy resistance and recurrence in ovarian cancer patients. In this regard, it has been hypothesized that disseminating epithelial cancer cells may undergo a variety of biochemical changes and reversibly acquire fibroblastoid or mesenchymal traits, known as epithelial-to-

mesenchymal-transition (EMT), which has already been described for breast cancer CTCs [20, 21]. EMT occurs under physiological conditions, however, is also a key mechanism for malignant progression. “Oncogenic EMT” allows tumor cells to acquire invasive properties and to develop metastatic growth characteristics. Moreover, it protects them from hostile conditions during the dissemination process. Interestingly, disseminated tumor cells may revert to their original epithelial phenotype, referred to as mesenchymal-epithelial-transition (MET), which promotes their colonization and contributes to the establishment of ultimately metastatic sites [22-29].

The PI3K/Akt/mTOR signalling pathway is aberrantly activated in the majority of human malignancies and confers oncogenic functions by promoting proliferation and cell survival [30, 31]. Therefore, this pathway has attracted widespread attention as therapeutic target for several malignancies [32]. Interestingly, recent evidence suggested that the PI3K/Akt/mTOR pathway is also essentially involved in EMT-regulation, thereby promoting tumor aggressiveness [33]. Moreover, repression of the cellular adhesion molecule E-cadherin, which is considered a hallmark of EMT, is mediated by EMT-associated transcription factors, such as Twist, Snail, Slug or Zeb [34]. Of those, especially Twist raised considerable attention, since it can also mediate invasiveness, drug resistance and EMT through a positive feedback loop with Akt, therefore providing a direct link to the PI3K/Akt/mTOR pathway [33].

In ovarian cancer, aberrant activation of the PI3K/Akt/mTOR pathway has also been reported and EMT is supposed to promote chemo-resistance [30, 35]. Interestingly, it has been suggested that cisplatin treatment of ovarian cancer cells generates residual cells with EMT-like traits [36]. However, it is still actively discussed, whether ovarian cancer cells in the primary tumor actually undergo a complete transition to a mesenchymal state [37]. Moreover, it is unknown, whether EMT-associated phenotypes extend to CTCs in the blood of ovarian cancer patients and whether they contribute to the heterogeneity of ovarian cancer CTCs. Therefore, assuming that dissemination of ovarian cancer cells requires at least a partial shift to a mesenchymal phenotype, the main purpose of our study was to analyze the incidence of epithelial and EMT-like CTCs at primary diagnosis of ovarian cancer. Moreover, we investigated how their detection rate is influenced by platinum-based chemotherapy. As a secondary objective, we analyzed EMT-associated transcript markers in more detail and were interested, how particular molecular phenotypes of EMT-like CTCs respond to platinum-based chemotherapy.

RESULTS

Platinum-based chemotherapy selects for EMT-like CTCs in ovarian cancer

We analyzed the epithelial associated marker transcripts EpCAM, Muc-1 and CA-125 as well as the representative EMT-associated marker transcripts PI3Kα, Akt-2 and Twist before surgery (n = 91) and in paired blood-samples after platinum-based chemotherapy (n = 31). Positivity for each CTC-subtype was defined by the detection of at least one of the transcripts of each marker panel, respectively. Epithelial CTCs were detected with an overall incidence of 18% before surgery, which slightly decreased to 14% after platinum-based chemotherapy. EMT-like CTCs were observed with a considerably higher detection rate at baseline (30%) and their incidence further increased after chemotherapy to an overall detection frequency of 52% (Figure 1, Supplementary Table 1), suggesting that platinum-based chemotherapy selects for EMT-like CTCs.

Epithelial and EMT-like CTCs exhibit a low phenotypic overlap

Having described the incidence of epithelial and EMT-like CTCs by separate analyses, we now investigated the overlap between epithelial and EMT-associated phenotypes (Figure 2, Supplementary Table 1). Before surgery, patients with detectable CTCs were positive for either EMT-associated transcripts (58%) or epithelial-associated transcripts (24%). Thus, epithelial and EMT-like CTCs were mutually exclusive in the majority of patients (82% in total). Interestingly, only a minor fraction of patients showed up with dual positivity of epithelial and EMT-like CTCs (18%), indicating that epithelial- and EMT-like CTCs seem to represent mostly independent CTC populations with low phenotypic overlap. After chemotherapy, this trend was retained and the proportion of exclusively EMT-positive CTCs further increased up to 76%, whereas the number of patients with exclusively epithelial CTCs or dual positivity each declined to 12%.

PI3Kα and Twist positive EMT-like CTCs are specifically enriched by platinum-based chemotherapy

We were further interested in the molecular phenotypes of epithelial and EMT-like CTCs in ovarian cancer and their response to platinum-based chemotherapy. Figure 3A illustrates the marker distribution among epithelial CTCs. Detection frequencies for EpCAM and Muc-1 were calculated independently from each other and in reference to only those patients with positivity for epithelial CTCs. Before surgery, positivity for epithelial CTCs mostly resulted from Muc-1 positivity, which was detected in 81% of cases, whereas EpCAM transcripts were less frequently detected (38%). After chemotherapy, detection frequency was slightly reduced for both marker transcripts; however, Muc-1 positivity remained three times more abundant than EpCAM-positivity (75% Muc-1-positive vs. 25% EpCAM-positive, Supplementary Table 1).

Figure 3B depicts the molecular heterogeneity of epithelial CTCs in more detail. Percentages were calculated in reference to only those patients with positivity for epithelial CTCs, now also considering dual positivity. Before surgery, the majority of epithelial CTCs were exclusively Muc-1 positive (62%), whereas only 19% were exclusively EpCAM-positive and further 19% showed dual positivity for Muc-1 and EpCAM. After chemotherapy, dual EpCAM+Muc-1-positive CTCs were no longer detectable and only Muc-1 (75%) or EpCAM (25%) positive CTCs were observed. CA-125 transcripts could not be detected at any time in our patient cohort, indicating expression levels below the detection limit of our assay or complete absence of CA-125 transcripts in the enriched CTC populations (Supplementary Table 1).

Subsequently, we performed the same kind of analysis for the EMT-associated marker panel (Figure 3C). Here again, percentages for PI3Kα, Akt-2 and Twist were calculated independently from each other in reference to only those patients with positivity for EMT-like CTCs. Before surgery, PI3Kα was observed in 35% of patients with positivity for EMT-like CTCs and Akt-2 in 46% of patients. Twist was most frequently detected (54%, Supplementary Table 1). Interestingly, after platinum-based chemotherapy, positivity rates for PI3Kα and Akt-2 slightly decreased, whereas Twist positivity was substantially elevated up to 69% in post-therapeutic blood samples, which is in accordance to the overall increase of EMT-like CTC (Figure 1).

Figure 3D depicts the molecular heterogeneity of EMT-like CTCs in more detail. Percentages were calculated in reference to only those patients with positivity for EMT-like CTCs, now also considering dual or triple positivity. Before surgery, exclusively Twist positive CTCs were most abundant (42%) followed by Akt-2 (19%) and PI3Kα-positive CTCs (12%). Additional CTC-phenotypes with dual or triple positivity for EMT-markers were observed with low or moderate detection frequency (15% PI3Kα+Akt-2 / 4% Akt-2+Twist / 8% PI3Kα+Akt-2+Twist). After chemotherapy, selective changes in the composition of molecular CTC-phenotypes became obvious. Interestingly, an additional molecular CTC-phenotype with dual PI3Kα+Twist positivity emerged, mostly, at the expense of PI3Kα+Akt-2-positive CTCs (Supplementary Table 1). The proportion of the other CTC-phenotypes remained largely stable in response to platinum-based chemotherapy. Notably, while the proportion of exclusively Twist or exclusively PI3Kα positive CTCs also remained nearly unchanged in post-chemotherapeutic blood samples, we conclude that the increase in the overall incidence of EMT-like CTCs (as shown in Figure 1) is accompanied by the “de novo” emergence of a dual PI3Kα+Twist positive CTCs after chemotherapy.

Clinical relevance of EMT-like CTCs

We inquired, whether the presence and enrichment of EMT-like CTC subtypes correlates with the patient’s clinicopathological parameters or with their survival. We observed the trend that patients with a residual tumor burden after primary debulking surgery were more likely to have EMT-like CTCs in their blood after adjuvant chemotherapy than patients with a macroscopically complete tumor resection. This association became statistically significant, after excluding patients with distant metastasis (FIGO IV; p=0.02).

Subsequently, we investigated prognostic significance of epithelial and EMT-like CTCs before surgery and after chemotherapy by Kaplan-Meier analysis. In the unselected total patient cohort, no prognostic relevance of epithelial CTCs before surgery or after chemotherapy could be demonstrated (Supplementary Figures 3). However, after excluding patients with FIGO IV as possible confounders, which per se have a poor prognosis, the presence of epithelial CTCs at primary diagnosis significantly indicated decreased PFS (HR: 2.63, 95% CI: 1.24-15.53; p=0.027) and OS (HR: 5.79, 95% CI: 2.98-162.7; p=0.003, Figure 4A+B). There was no prognostic significance of EMT-like CTCs before surgery or after chemotherapy in the total patient cohort (Supplementary

Figures 4). However, combined analysis showed that the presence of epithelial CTCs or PI3Kα transcripts indicates reduced OS (HR: 3.25, 95% CI: 1.31-15.47; p=0.018, Figure 4C). Interestingly, this finding could be confirmed with increased statistical significance, after excluding FIGO IV patients, and the presence of epithelial CTCs or PI3Kα transcripts patients indicated reduced PFS (HR: 2.35, 95% CI: 1.06-8.74; p=0.042) and OS (HR: 7.22, 95% CI: 3.21-111.5; p=0.001, Figure 4D+E).

DISCUSSION

In the present investigation, we analyzed incidence and molecular phenotypes of EMT-like CTCs in the blood of ovarian cancer patients and monitored their response to platinum-based chemotherapy. EMT-like CTCs were, already at primary diagnosis, more abundantly detected than epithelial CTCs and showed low phenotypic overlap with epithelial CTCs. After chemotherapy, we observed a selective enrichment of EMT-positive CTCs, which was accompanied by the “de novo” emergence of dual PI3Kα and Twist positive CTCs.

At present, there is no standard definition for the identification of CTCs and a variety of CTC-enrichment and detection strategies are available, based on CTC-associated surface antigens or intrinsic (physical or tumor-biological) CTC properties, such as size, deformability, invasive capacity or telomerase activity [38, 39]. Subsequent CTC-detection can be carried by a broad spectrum of methods, such as immunocytological or molecular biology based assays [10, 11, 39-43]. In line with our previous investigations [10, 15], we took advantage of the AdnaTest *OvarianCancer*, which allows a more detailed molecular characterization of enriched CTCs. Notably, overall detection frequency of epithelial CTCs at primary diagnosis, reported herein, was comparable to our previous analyses, confirming comparability of the underlying study with our previous observations on larger patient cohorts [10]. In the present investigation, we were interested in the incidence and dynamics of EMT-associated CTC-phenotypes, which, to the best of our knowledge, has never been investigated in ovarian cancer patients so far.

We observed that EMT-associated marker transcripts considerably expanded the phenotypic range of CTC-detection. Already at primary diagnosis, EMT-like CTCs were more abundant than epithelial CTCs. This is in accordance with findings on breast cancer, reporting that EMT is a rare event in the primary tumor, however, frequently

occurs among CTCs [21, 44]. Therefore, we conclude that EMT is also a common event among ovarian cancer CTCs and might already have been initiated in the primary tumor. Interestingly, recent data from a pancreatic cancer mouse model even suggested that EMT-like CTCs can already be shed into the circulation from pre-invasive lesions [45].

We explicitly used the term EMT-“like” CTCs in our study and strictly avoiding any other descriptive terms that might imply that these CTCs already acquired a fully mesenchymal state. Since the immunomagnetic enrichment of CTCs in our assay is based on the epithelial surface epitopes MUC and EpCAM, this assay cannot detect fully mesenchymal CTCs, which have completely downregulated their epithelial surface epitopes. Thus, EMT-like CTCs, selected and characterized herein, express the epithelial marker proteins EpCAM and MUC on their surface, allowing immunomagnetic selection, however, do not express EpCAM or Muc-1 on transcript level above the detection limit of our assay. At a first glance, this may appear counterintuitive, however, discordances between protein and transcript expression profiles of a cell can be explained by post-transcriptional modifications of messenger RNA or differences in the half-live time between messenger RNA and their corresponding proteins [46-48]. Notably, since these CTCs also co-express Akt-2, PI3Kα or Twist, we describe snapshots of “semi-mesenchymal” CTC [49]. Semi-mesenchymal CTCs could be either on their way to an ultimately mesenchymal phenotype (EMT) or on their way back to an epithelial phenotype (MET) or, alternatively, they could persist in this intermediate state. Although the biology of semi-mesenchymal CTCs is largely unknown, we could speculate that particularly a semi-mesenchymal state reflects an aggressive CTC-phenotype with high degree of plasticity, which facilitates the adaption of CTCs to hostile environmental stimuli during dissemination. This is in line with the hypothesis, that EMT (and its reversion MET) is a highly dynamic process and describes different continuous phenotypes, rather than a dichotomous switch between epithelial and mesenchymal states. Notably, those phenotypic changes directly influence the yield of CTC-detection assays that are based on epithelial selection markers [49]. However, our assumption of continuous CTC phenotypes in ovarian cancer is not necessarily supported by the fact that we observed a trend for a mutual exclusion between patients with only epithelial CTCs and those with only semi-mesenchymal CTCs. This is a very interesting finding and may indicate that there is also a subgroup of ovarian cancer patients with “fully” epithelial CTCs,

without any shifts towards EMT. These CTCs may have entered the bloodstream via passive dissemination [50], however, clinical relevance of this finding requires further investigation. Applying our EMT-associated marker set, we revealed a heterogeneous spectrum of EMT-associated CTC-phenotypes, pointing to CTC-heterogeneity in ovarian cancer, which has already been reported for CTCs in a variety of other cancer entities, such as prostate or breast cancer [51, 52]. Notably we cannot distinguish with our assay, whether a co-expression of more than one marker-transcript is derived from CTCs, actually co-expressing these markers on a same cell, or from separate semi-mesenchymal CTC-populations, concomitantly present in the “pool” of enriched CTCs.

Oncogenic EMT has received considerable attention over the past years and is associated with cancer aggressiveness, metastasis and tumor cell plasticity [29]. Interestingly, we observed a clear increase of semi-mesenchymal CTCs in response to platinum-based chemotherapy, due to a shift towards PI3Kα and Twist expression. This finding is of particular clinical interest, since it has already been described that cancers may acquire resistance to systemic treatment as a result of clonal evolution and selection [53]. We therefore assume a clonal selection of CTCs with activated PI3Kα and Twist associated signaling pathways, which might be therapy refractory and could be responsible for recurrence of ovarian cancer. This assumption is strongly supported by our previous studies on breast cancer patients, in which we already showed that preferentially semi-mesenchymal and potentially platinum-resistant (ERCC1-expressing) CTCs remain after neoadjuvant chemotherapy [54]. Moreover, a further independent key publication reported on dynamic changes in the epithelial and mesenchymal composition of breast cancer CTCs in response to therapy. In this study, clinical response was accompanied by a switch to predominantly epithelial CTCs, whereas progressive disease correlated with the increase of mesenchymal CTC-phenotypes [21]. For ovarian cancer, *in vitro* experiments already suggested that cisplatin treatment of ovarian cancer cells generates residual cells with EMT-like traits [36]. Therefore, we assume that PI3Kα and Twist positive CTCs may reflect tumor evolution in response to platinum-based chemotherapy. Interestingly, this also suggests a link to recent studies on genomic tumor evolution, reporting on an increase in activating PIK3CA mutation among cell free tumor DNA of breast cancer patients, following paclitaxel treatment [53]. In this context, our findings could have several diagnostic or therapeutic implications, since PI3Kα and Twist are functionally involved in pathways controlling tumor cell survival or platinum-resistance [55, 56]. The most

important limitation of our study is the small number of patients, particularly when comparing the detection frequency of single transcript markers in pre- and post-therapeutic blood samples. Nevertheless, this detailed analysis, albeit only descriptive, was considerably informative for us and complemented our key finding, the enrichment of semi-mesenchymal CTC after platinum-based chemotherapy.

Among our exploratory survival analysis, we did not confirm prognostic relevance of epithelial CTCs in the unselected study population, which is in contrast to our previous finding and could be explained by the yet limited follow-up period of the present study [10]. Nevertheless, after excluding FIGO IV patients as possible confounders, prognostic relevance of epithelial CTCs could be restored. Apart from this, the presence of epithelial CTCs at primary diagnosis, in combination with PI3Kα-positivity, indicated poor prognosis not only in FIGO I-III patients, but also in the unselected cohort, suggesting that PI3Kα-positivity marks a clinically relevant subgroup of EMT-associated CTCs, which could be derived from the untreated primary tumor. In contrast, we reported that patients with residual tumor after primary debulking were more likely to be positive for EMT-like CTCs after adjuvant chemotherapy, indicating that this CTC-population could be disseminated directly from residual tumor burden under the selective pressure of chemotherapy, after conversion to a semi-mesenchymal state.

Conclusively, this is the first study on EMT-like CTCs in ovarian cancer, reporting that platinum-based chemotherapy provokes a shift of molecular phenotypes towards PI3Kα and Twist expressing CTCs, which may reflect clonal tumor evolution. Therefore, we encourage to further investigate the functional role of semi-mesenchymal CTCs in the malignant progression of ovarian cancer and to determine, whether these EMT-like CTCs can be a biomarker for high risk minimal residual disease in ovarian cancer. In this context, an extended multi-marker panel with mesenchymal and tumor stem cell-associated genes, which we recently established for metastatic breast cancer [18], could be a useful liquid biopsy tool, in order to detect a broad spectrum of CTC-phenotypes for therapy monitoring. Moreover, PI3Kα and Twist positive CTCs could be an attractive therapeutic target, since PI3K/Akt/mTOR pathway inhibitors are currently being investigated for ovarian cancer among several preclinical studies and also a few ongoing clinical trials [32] (NCT01623349, NCT02476955). Moreover, it was shown for ovarian cancer that low dose metformin, a first line drug for treating diabetes, can also reduce the expression of EMT-associated

makers, such as Twist, suggesting further options for potentially targeting this CTC-population [57]. Since the biology of ovarian cancer CTCs is largely unknown, we believe that our finding could be a step forward in understanding their heterogeneity and their dynamics in response to chemotherapy.

MATERIAL AND METHODS

Patient characteristics

The present study was conducted at the Departments of Gynecology and Obstetrics at the University Hospitals of Essen and Dresden, Germany. In this study, a total of 95 patients, diagnosed between 2010 and 2014 with histologically confirmed epithelial ovarian cancer, were analyzed. Clinical characteristics of the patients are documented in Table 1. Informed written consent was obtained from all patients and the study was approved by the Local Ethic Committees (Essen 05-2870; Dresden EK 236082012) and was performed according to the declaration of Helsinki. Tumors were classified according to the WHO classification of tumors of the female genital tract. Grading was conducted using the grading system proposed by Silverberg [58] and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique. The whole study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy and peritoneal stripping was performed. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if macroscopic complete tumor resection was achieved. All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m^2.

Enrichment and molecular characterization of ovarian cancer CTCs

Peripheral blood (5 ml) from each patient was collected in EDTA tubes (Sarstedt & AG+Co., Nümbrecht, Germany) and processed within 4h for the enrichment of CTCs and subsequent expression analysis, according to the AdnaTest *OvarianCancer Detect and* the AdnaTest *EMT-1 Detect* (QIAGEN, Hannover GmbH, Langenhagen, Germany, Supplementary Figure 1+2). These assays have been described in detail elsewhere [10, 15, 44]. The Adnatest was performed in biological replicates; therefore two independent consecutive blood samples were obtained from each patient at each time point. Briefly, we applied immuno-magnetical enrichment of CTCs (Adnatest

OvarianCancer Select, Adnatest EMT-1Select), targeting epithelial cellular adhesion molecules.

For the detection of epithelial CTCs, RNA was isolated and gene expression analysis was performed by reverse-transcription (RT) and multiplex RT-PCR, detecting EpCAM, Muc-1, and CA-125 (AdnaTest *OvarianCancerDetect).* In this assay, amplicons with the following sizes were generated: EpCAM: 396bp; Muc-1: 293bp; CA-125: 432bp. For the detection EMT-like CTCs, RNA was isolated and gene expression analysis was performed by reverse-transcription (RT) and multiplex RT-PCR, detecting PI3Kα, Akt-2 and Twist (AdnaTest *EMT-1Detect).* Contaminating leukocytes (about 1500 per sample) were reduced by approximately 10fold using a special washing buffer (*AdnaWash* buffer) enabling the proper differentiation of EMT expression profiles with a specificity and sensitivity of >90%, which was confirmed in healthy donor samples [20, 44]. In this assay, amplicons with the following sizes were generated: PI3Kα: 595bp, Akt-2: 306 bp; Twist: 203bp. β-actin served as an internal control (amplicon size: 119 bp) and PCR-products were visualized with the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). An amplicon concentration of >0.2 ng/µl was applied as threshold for EpCAM, Muc-1 or CA-125 positivity. Amplicon concentration of >0.2 ng/µl was applied as threshold for Akt-2, >0.25ng/µl for PI3Kα and >0,15ng/µl for Twist positivity, respectively.

The analytical sensitivity of the detection of CTC-associated EMT-transcripts was determined by the analysis of a low number of target cells (5 IGROV ovarian cancer cells spiked into 5 ml blood of healthy donors). Healthy donor samples without spiked tumor cells were used to determine the specificity of the test. Applying the above mentioned amplicon cut-off values, 97% of 30 healthy donor samples were negative for EMT markers. These experiments demonstrate that a potential risk of false-positive events is negligible in our present analysis.

ABBREVIATIONS

BM	bone marrow
CTC(s)	circulating tumor cell(s)
DTC(s)	disseminated tumor cell(s)
EMT	epithelial-to-mesenchymal-transition
EpCAM	epithelial cell adhesion molecule
ERCC1	excision repair cross-complementation group 1
MET	mesenchymal-to-epithelial-transition
Muc-1	Mucin-1
OS	overall survial
PFS	progression free survival
RT	reverse transcription

AUTHOR CONTRIBUTIONS

IC, SKB, PB, PW, SH, RK and JDK made substantial contributions to the conception and design of the study, to the experimental work or to the acquisition of data and to the analysis/interpretation of the results. JDK, SKB, IC, PW and SH were involved in drafting the manuscript or revising it. All authors read and approved the manuscript in its final version.

CONFLICT OF INTEREST STATEMENT

Sabine Kasimir-Bauer is a consultant for QIAGEN, Hilden, Germany.

Siegfried Hauch is an employee of QIAGEN, Hilden, Germany.

All other authors declare that they have no conflict of interest.

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Tab.1 Patient characteristics at the time of primary diagnosis

Total	**95**
Age	median 61 years, (31-82) years
FIGO stage	
I-II	16 (17%)
III	61 (64%)
IV	18 (19%)
Nodal status	
N_o	33 (37%)
N_1	32 (43%)
N_x	30 (20%)
Grading	
I-II	32 (43%)
III	63 (57%)
Residual tumor	
Macroscopic complete resection	48 (51%)
Any residual tumor	40 (42%)
Unknown	7 (7%)
Histologic type	
Serous	81 (80%)
Mucinous	5 (14%)
Other	9 (6%)
Survival	
PFS[1]	median 6 months, (0-51 months)
OS[2]	median 21 months, (1-59 months)
Alive	15 (16%)
Dead	72 (76%)
Unknown	8 (8%)
Recurrence	
No relapse	50 (43%)
Relapse	28 (55%)
Unknown	17 (2%)

[1]PFS: progression-free survival, [2]OS: overall survival

FIGURE LEGENDS

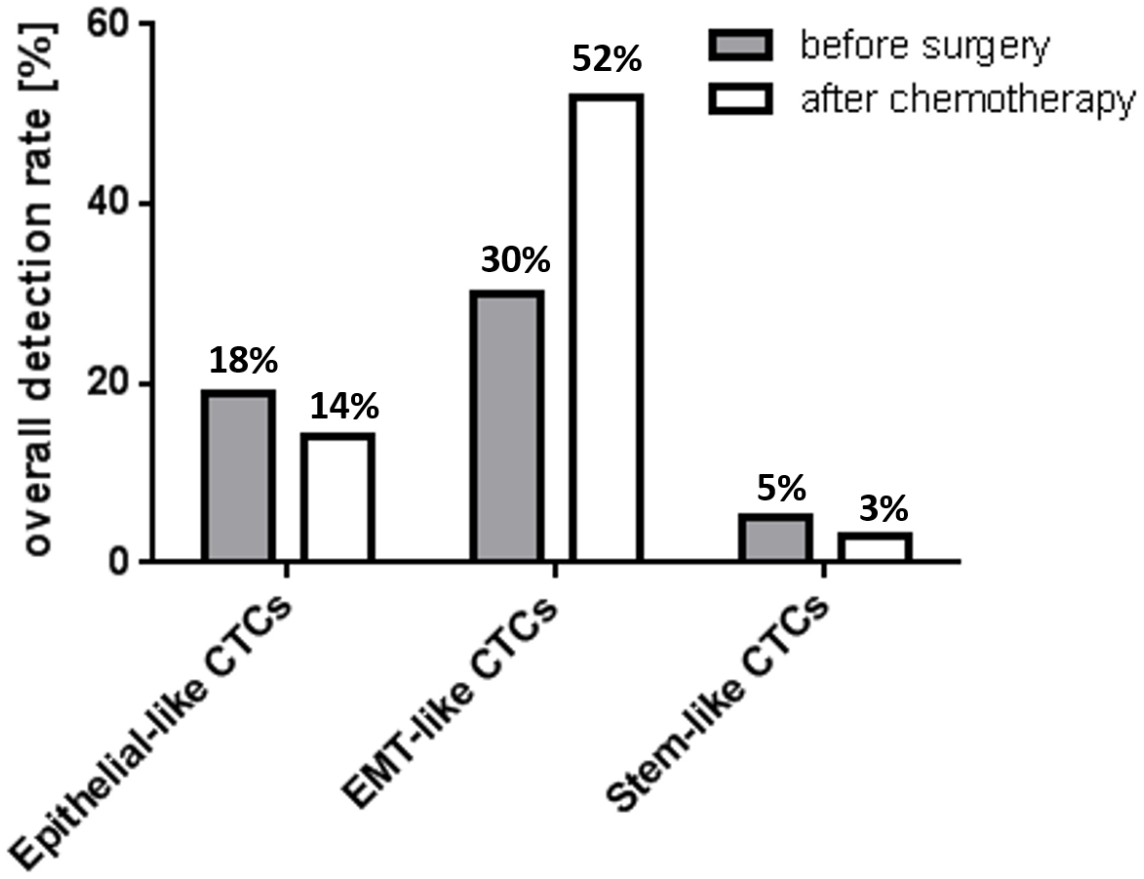

Fig.1: Overall detection frequency of epithelial and EMT-like CTCs in ovarian cancer

The bar chart illustrates overall detection rates of epithelial and EMT-like CTCs in ovarian cancer patients before surgery and after chemotherapy. Percentages for the two classes of CTCs were calculated independently from each other and, in both cases, refer to the whole study population (before surgery: n=91, after chemotherapy n=31). A patient was considered “epithelial CTC-positive” or “EMT-like CTC-positive”, if one of the epithelial markers or one of the EMT-associated markers was detectable, respectively.

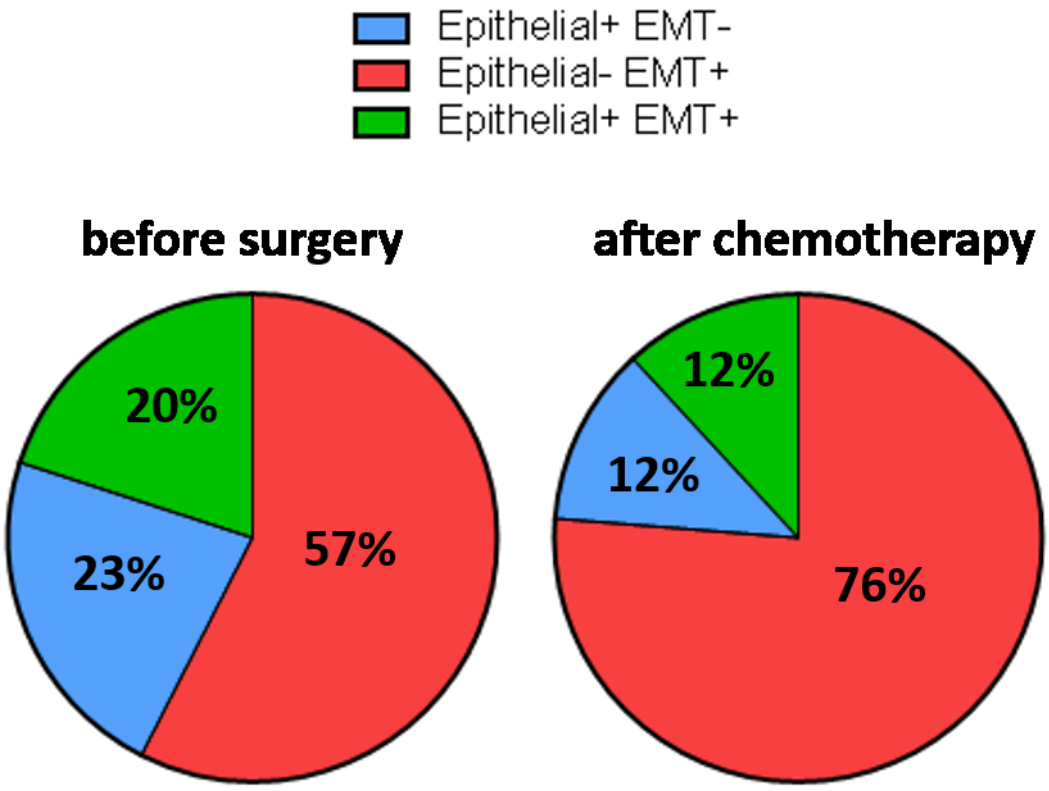

Fig.2: Phenotypic overlap of epithelial and EMT-like CTCs

The pie chart depicts the overlap of epithelial and EMT-like CTCs in ovarian cancer patients before surgery and after chemotherapy. Percentages were calculated in reference to all patients with overall CTC-positivity. Besides patients with exclusively epithelial (blue) and exclusively EMT-like CTCs (red), there were also patients, harbouring both CTC populations in their blood (green).

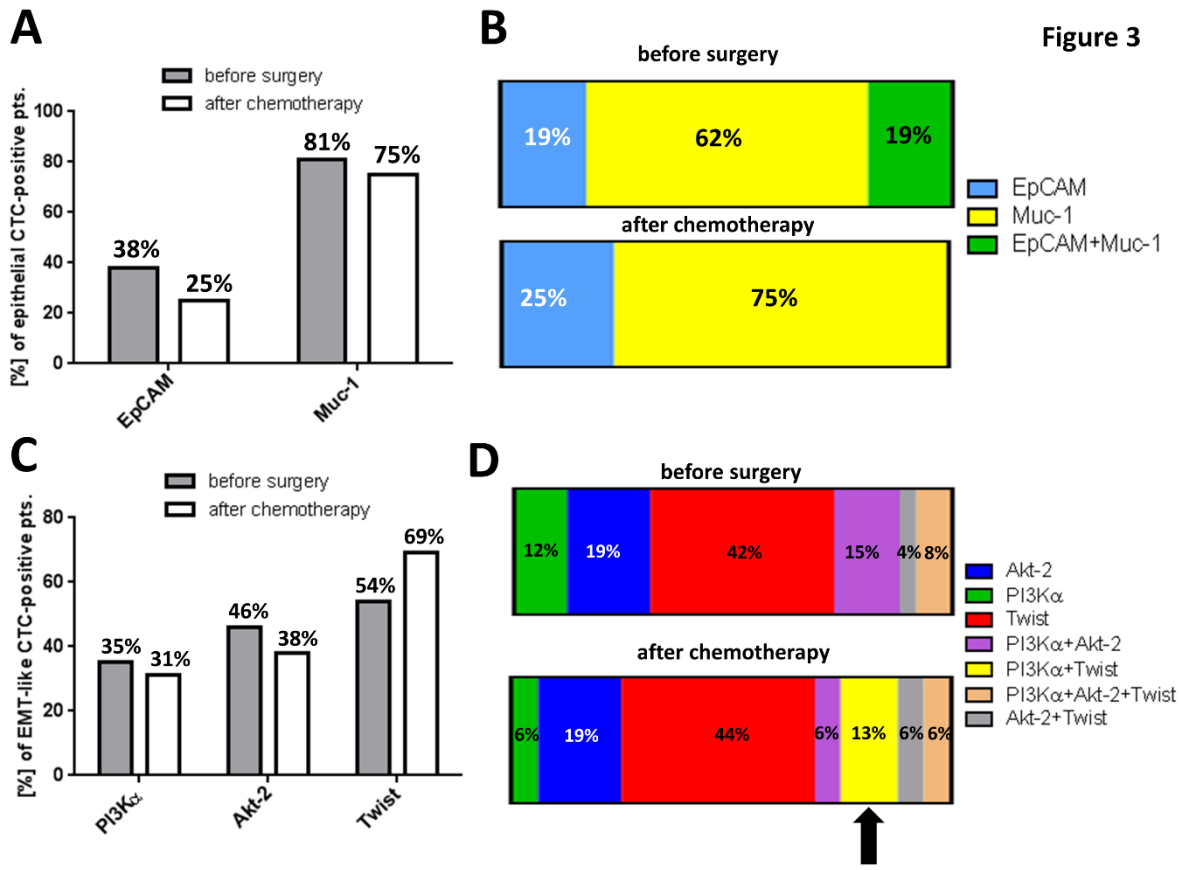

Figure 3

Fig.3: Heterogeneity of EMT-associated CTC-phenotypes and their response to platinum-based chemotherapy

The figure summarizes molecular phenotypes of EMT-like CTCs and their response to platinum-based chemotherapy. **A** The bar chart shows the marker distribution in epithelial CTC-positive patients. Percentages for EpCAM and Muc-1 were calculated independently from each other and in reference to only those patients with positivity for epithelial CTCs. **B** The stacked bar chart illustrates the marker distribution of epithelial CTC-positive patients, also considering dual-positivity for EpCAM and Muc-1. **C** The bar chart shows the marker distribution in EMT-like CTC-positive patients. Percentages for PI3Kα Akt-2 and Twist were calculated independently from each other and in reference to only those patients with positivity for EMT-like CTCs. **D** The stacked bar chart illustrates the marker distribution in EMT-like CTC-positive patients, now also considering dual- or triple positivity for EMT-associated transcripts.

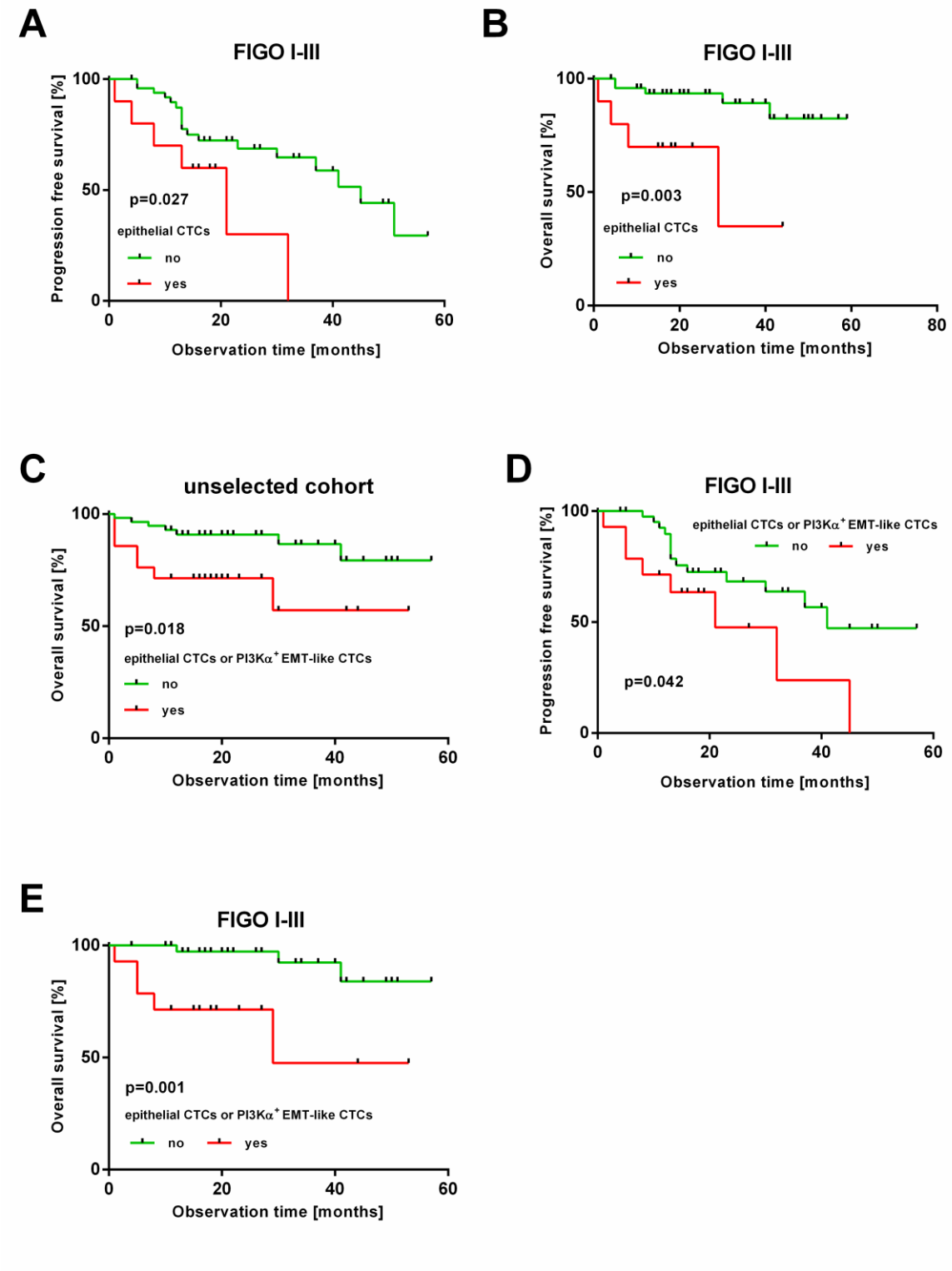

Fig.4: Prognostic relevance of epithelial and EMT-like CTCs

The Kaplan-Meier plots show prognostic relevance of different CTC-subtypes: **A+B** epithelial CTCs in FIGO I-III patients without distant metastasis **C** epithelial or PI3Kα-positive CTCs in the unselected patient cohort **D+E** epithelial or PI3Kα-positive CTCs in FIGO I-III patients. Red curves represent patients, positive for the respective CTC-subtype, green curves show CTC-negative patients.

Suppl Tab.1: Distribution of epithelial and EMT-associated transcripts in CTCs of ovarian cancer patients

Before Surgery				After Chemotherapy			
Figure 1	**Number of positive patients**	**n**	**%**		**Number of positive patients**	**N**	**%**
epithelial CTCs	16	91	**18**	epithelial CTCs	4	29	**14**
EMT-like CTCs	26	86	**30**	EMT-like CTCs	16	31	**52**
Figure 2							
epithelial CTC-positive/ EMT-like CTC-positive	6	34	**18**	epithelial CTC-positive/ EMT-like CTC-positive	2	17	**12**
epithelial CTC-positive/ EMT-like CTC-negative	8	34	**24**	epithelial CTC-positive/ EMT-like CTC-negative	2	17	**12**
epithelial CTC-negative/ EMT-like CTC-positive	20	34	**59**	epithelial CTC-negative/ EMT-like CTC-positive	13	17	**76**
Figure 3a							
EpCAM	6	16	**38**	EpCAM	1	4	**25**
Muc-1	13	16	**81**	Muc-1	3	4	**75**
Figure 3b							
EpCAM	3	16	**19**	EpCAM	1	4	**25**
Muc-1	10	16	**63**	Muc-1	3	4	**75**
Figure 3c							
PI3Kα	9	26	**35**	PI3Kα	5	16	**31**
AKT	12	26	**46**	AKT	6	16	**38**
Twist	14	26	**54**	Twist	11	16	**69**
Figure 3d							
PI3Kα	3	26	**12**	PI3Kα	1	16	**6**
AKT	5	26	**19**	AKT	3	16	**19**
Twist	11	26	**42**	Twist	7	16	**44**
PI3Kα +Akt-2	4	26	**15**	PI3Kα +Akt-2	1	16	**6**
PI3Kα +Twist	0	26	**0**	PI3Kα +Twist	2	16	**13**
Akt-2+Twist	1	26	**4**	Akt-2+Twist	1	16	**6**
PI3Kα +Akt-2+Twist	2	26	**8**	PI3Kα +Akt-2+Twist	1	16	**6**

The table summarizes the absolute counts and calculated positivity rates of patients with different CTC-subgroups, according to epithelial and EMT-associated CTCs. Subgroups are listed as they chronologically appear in Figure 1-3 in the manuscript. According to Figure 1, a patient was counted positive for epithelial or EMT-like CTCs, as soon as at least one of the epithelial or EMT-associated markers was detected in one of the two biological replicate samples. According to Figure 2, co-incidence of positivity for epithelial and EMT-like CTCs was assessed. In Figure 3a and Figure 3c, percentages for (EpCAM or Muc-1) or (PI3Kα or Akt-2 or Twist) were calculated, independently from each other, and in reference to only those patients with positivity for epithelial or EMT-like CTCs, respectively. In Figure 3b and 3d, percentages were calculated in reference to patients with positivity for epithelial or EMT-like CTCs, respectively, also considering dual or triple marker positivity.

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R*ASSF1A* promoter methylation in high-grade serous ovarian cancer: a direct comparison study in primary tumors, adjacent morphologically tumor cell free tissues and paired circulating tumor DNA

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KEY WORDS

Circulating tumor DNA; *RASSF1A*; high-grade serous ovarian cancer; methylation specific PCR; High Resolution Melting Analysis

ABSTRACT

The *RASSF1A* promoter is frequently methylated in high-grade serous ovarian cancer (HGSC). We examined *RASSF1A* promoter methylation in primary tumors, adjacent morphologically tumor cell-free tissues and corresponding circulating tumor DNA (ctDNA) samples of patients with HGSC, using a real-time methylation specific PCR (real-time MSP) and a methylation-sensitive high-resolution melting analysis (MS-HRMA) assay for the detection and semi-quantitative estimation of methylation, respectively. Two groups of primary HGSC tumor FFPE samples were recruited (Group A n=67 and Group B n=61), along with matched adjacent morphologically tumor cell-free tissues (n=58) and corresponding plasma samples (n=59) for group B. Using both assays, *RASSF1A* promoter was found highly methylated in primary tumors of both groups, and at lower percentages in the adjacent morphologically tumor cell-free tissues. Interestingly, *RASSF1A* promoter methylation was also observed in ctDNA by real-time MSP. Overall survival (OS) was significantly associated with *RASSF1A* promoter methylation in primary tumor samples using MS-HRMA (P=0.023). Our results clearly indicate that *RASSF1A* promoter is methylated in adjacent tissue surrounding the tumor in HGSC patients. We report for the first time that *RASSF1A* promoter methylation provides significant prognostic information in HGSC patients.

INTRODUCTION

Ovarian cancer represents the third most frequent gynecological cancer and the fifth leading cause of cancer-related death in women [1]. Epithelial ovarian cancer is the main type, characterized by histological and molecular heterogeneity. The most common subtype, high-grade serous ovarian cancer (HGSC), is often diagnosed at an advanced stage and little progress has been achieved in standard treatment and overall survival (OS) during the last three decades [2]. Primary disease is treated with surgical removal of the tumor, followed by a combination of platinum and taxane-based chemotherapy [3, 4] with about 20% of patients found to be resistant to this treatment [5, 6]. New multimodal therapeutic concepts now include targeted therapy applying Bevacizumab or the PARP inhibitor Olaparib in certain clinical situations [7, 8].

It is now clear that epigenetic alterations hold an important role in cancer initiation and progression and that aberrant DNA methylation, especially promoter hypermethylation of tumor suppressor genes is a frequent event in most human cancers [9]. Epigenetic inactivation of a tumor suppressor gene often results from its promoter methylation and is considered as an early event during carcinogenesis [10]. Many studies have reported methylation changes in epithelial ovarian cancer [11] and a recent review summarizes the differences in the observed methylation patterns in the main histological subtypes of the disease, including HGSC [12].

Cell-free DNA (cfDNA) circulates at high concentrations in cancer patients and can be used for the detection of several molecular alterations related to cancer development [13]. Circulating tumor DNA (ctDNA) is a small percentage of cfDNA that is shed in circulation by tumor cells and carries all these molecular alterations including tumor specific mutations, microsatellite instability (MI) [13], loss of heterozygosity (LOH) [14], and DNA methylation [15]. Circulating tumor DNA is a very promising non-invasive diagnostic, prognostic and predictive tool, since it provides an easily accessible source of DNA derived from the tumor [16]. Our group has reported *SOX17* [17, 18], *CST6* [19] and *BRMS1* [20] promoter methylation in cfDNA in breast and non-small cell lung cancer patients.

The *RASSF1* gene belongs to the Ras-association domain family that consists of ten members. RASSF proteins contribute to microtubule stability and they are involved in cell cycle regulation, apoptosis, cell migration and cell adhesion. The *RASSF1* gene is found on the 3p21.3 locus and comprises eight exons. Its two promoter regions and

the implied alternative splicing are responsible for the eight isoforms A-H. *RASSF1A* and *RASSF1C* are mostly studied so far, especially *RASSF1A* gene isoform that definitely acts as a tumor suppressor in human cancer [21, 22]. *RASSF1A* is involved in molecular pathways including Ras/PI3K/AKT, Ras/RAF/MEK/ERK, Hippo pathways and β-catenin signaling pathway [22, 23]. The *RASSF1A* gene is frequently inactivated by aberrant promoter hypermethylation in the majority of human malignancies, including breast, lung, gastrointestinal, bladder, head and neck cancer and gynecological cancers, endometrial and cervical cancer [23]. In ovarian cancer, *RASSF1A* promoter methylation has been identified in many studies [24], but no significant association with clinical outcome has been reported so far.

The aim of the present study was to examine the prognostic significance of *RASSF1A* promoter methylation in primary tumors, matched adjacent morphologically tumor cell-free tissues surrounding the tumor and the corresponding plasma samples of patients with HGSC. To evaluate the clinical significance of *RASSF1A* promoter methylation in HGSC, we applied a highly sensitive real-time methylation specific PCR (real-time MSP) assay [25] for the detection of *RASSF1A* promoter methylation and compared it to a methylation-sensitive high-resolution melting analysis (MS-HRMA) assay. We further directly compared *RASSF1A* promoter methylation between primary tumors, matched adjacent tissues and corresponding plasma ctDNA. To the best of our knowledge, this is the first study on the evaluation of *RASSF1A* promoter methylation status in HGSC that is based on matched primary tumors, adjacent tissues and corresponding plasma samples from the same patients. Our results clearly indicate that the *RASSF1A* promoter is methylated in adjacent tissue surrounding the tumor in HGSC patients. We also report for the first time that *RASSF1A* promoter methylation provides significant prognostic information in HGSC patients.

RESULTS

A schematic diagram of our study is shown in **Figure 1**.

RASSF1A promoter methylation status in HGSC by real-time MSP

RASSF1A promoter methylation status was first evaluated in the group A by real-time MSP. According to our results, *RASSF1A* promoter was methylated in 27/67 (40.3%) primary tumor samples. *RASSF1A* promoter methylation status was further evaluated in the group B. According to our results, *RASSF1A* promoter was methylated in 25/61 (41.0%) primary tumor samples. In the group of adjacent morphologically tumor cell-free tissues of group B, 17/58 (29.3%) samples were found methylated. In cfDNA, isolated from corresponding plasma, 15/59 (25.4%) samples were found positive for *RASSF1A* promoter methylation.

Semi-quantitative estimation of *RASSF1A* promoter methylation by MS-HRMA

We further evaluated the percentages of *RASSF1A* promoter methylation in primary tumor samples and adjacent tissues, by using the semi-quantitative MS-HRMA assay. *RASSF1A* promoter was found methylated in 27/67 (40.3%) primary tumor samples of group A and in 28/61 (45.9%) primary tumor samples of group B. 21/58 (36.2%) adjacent morphologically tumor cell-free tissues of group B were found methylated. The MS-HRMA assay can detect heterogeneous methylation; we found heterogeneously methylated samples both in group A (8/67, 11.9%) and in tumor samples of group B (7/61, 11.5%). We also observed heterogeneous methylation in 5/58 (8.6%) adjacent tissues of group B. According to the semi-quantitative MS-HRMA, in most positive cases *RASSF1A* promoter methylation was detected at a lower percentage in the adjacent morphologically tumor cell-free tissues, when compared to the paired primary tumors (**Figure 2**). However, there were three cases where the percentage of *RASSF1A* promoter methylation was higher in the adjacent tissue (**Figure 2**). No significant difference was observed (P=0.126, Mann-Whitney U test).

Comparison between real-time MSP and MS-HRMA

When we compared our results derived for the same primary tumor samples in both group A and group B, by real-time MSP and MS-HRMA, the agreement between the two assays was almost perfect (**Table 1**). More specifically, in the group A, there was an agreement for 63/67 (94.0%) primary tumor samples (P<0.001, 2-sided Pearson χ^2 test, k=0.876), while in the group B, there was an agreement for 58/61 (95.1%)

samples (P<0.001, 2-sided Pearson χ^2 test, k=0.900). In the group of adjacent tissue samples (group B), the agreement between real-time MSP and MS-HRMA was substantial (50/58, 86.2%, P<0.001, 2-sided Pearson χ^2 test, k=0.689) (**Table 1**).

Direct comparison of *RASSF1A* promoter methylation status in primary tumors, adjacent tissues and plasma ctDNA

We further directly compared *RASSF1A* promoter methylation status in 53 cases, where primary tumors, adjacent tissues and corresponding plasma ctDNA were available (triplets, n=53). *RASSF1A* promoter methylation status in primary tumors and adjacent tissues was evaluated using both real-time MSP and MS-HRMA, while in corresponding plasma samples only real-time MSP was used because of its higher sensitivity. In most cases there was a concordance between our findings in primary tumors, adjacent tissues and plasma (**Table 2)**. In 45/53 (84.9%) cases we found an agreement for *RASSF1A* promoter methylation between primary tumor samples and adjacent tissues (P<0.001, 2-sided Pearson χ^2 test, Cohen's kappa=0.674). According to the guidelines for the interpretation of k values, there is a substantial agreement between the two subgroups. In 33/53 (62.3%) cases we observed a slight agreement for *RASSF1A* promoter methylation between primary tumor samples and corresponding plasma, (P=0.227, 2-sided Pearson χ^2 test, k=0.156) (**Table 2**). In group B, we used again the 53 triplets for the comparison between primary tumor samples and adjacent tissues using MS-HRMA. The agreement between the two subgroups was 47/53 (88.7%, P<0.001, 2-sided Pearson χ^2 test, Cohen's kappa=0.768, substantial agreement).

Our results on *RASSF1A* promoter methylation in primary tumors, adjacent tissues and corresponding plasma samples are shown in **Figure 3**. In six patients, *RASSF1A* promoter methylation was detected in the primary tumor and in the adjacent tissue by both assays and in corresponding cfDNA in plasma by real-time MSP. In five patients, *RASSF1A* promoter methylation was detected only in plasma, while the primary tumors and adjacent tissues were found negative by both assays.

Prognostic significance of *RASSF1A* promoter methylation in HGSC

We further proceeded to the estimation of the clinical significance of *RASSF1A* promoter methylation status for the patients of group B, as Overall Survival (OS) and Progression-Free Survival (PFS) data were available along with other clinicopathological characteristics. The total number of patients is now different (n=47),

because in some cases the clinical information was not available and all cases where OS≤4 months were excluded from the survival study. The median OS was 36 months while the median PFS was 12.5 months (starting date being the date of diagnosis; PFS was estimated based on the date of relapse; OS was estimated based on the date of death). The correlation between *RASSF1A* promoter methylation status of primary tumor samples with clinicopathological features of the patients is shown in **Table 3**. *RASSF1A* promoter methylation was significantly associated with tumor grade using both assays (real-time MSP: P=0.043, MS-HRMA: P=0.037) and regional lymph nodes (pN) using MS-HRMA (P=0.040). No significant correlations are found between *RASSF1A* methylation status of adjacent tissues and plasma samples, and clinicopathological characteristics (data not shown).

The Kaplan-Meier analysis was further performed to correlate OS and PFS data with *RASSF1A* promoter methylation status. In primary tumor samples, OS was found to be significantly correlated with *RASSF1A* promoter methylation status using MS-HRMA (P=0.023, log-rank test, **Figure 4**), whereas no significant correlation was observed using real-time MSP (P=0.157, log-rank test). No significant correlations were found between OS and *RASSF1A* promoter methylation status of adjacent tissues and plasma samples, and between PFS and *RASSF1A* promoter methylation status of all three subgroups (data not shown).

Finally, *RASSF1A* promoter methylation in primary tumor samples and all the available clinicopathological features were tested in univariate Cox Regression analysis for association with OS and PFS. *RASSF1A* promoter methylation status using MS-HRMA and platinum resistance were significantly associated with decreased OS (P=0.030 and P=0.019, respectively). The lack of correlation between OS and clinical parameters known to be of predictive value, like age and tumor rest, can be possibly explained by the relatively small cohort analyzed here. We next performed multivariate Cox Regression analysis for *RASSF1A* promoter methylation estimated with the MS-HRMA assay and platinum resistance in association with OS, but no independent prognostic significance was observed. The results are shown in detail in **Table 4**.

DISCUSSION

RASSF1A promoter methylation is a common event in ovarian cancer, and was first identified in ovarian tumor samples over a decade ago [26-28]. Apart from primary tumors, benign cystadenomas and low malignant potential tumors exhibit *RASSF1A* promoter methylation as well [29, 30]. Choi et al. first tried to correlate *RASSF1A* promoter methylation with patients outcome, but did not find any correlation [30]. A possible explanation could be that in this study, all the ovarian cancer samples of serous histotype were concerned as a single cohort without taking into account the two subtypes, high- and low-grade serous ovarian cancer. However, it is now known that these two subtypes differ in the progenitor area and the tumors molecular profile [31]. High methylation frequency of *RASSF1A* has also been observed by Montavon et al. at HGSC tumor samples, however, the relatively small number of available survival data (n=37) could be a possible explanation for the lack of association between *RASSF1A* methylation and overall survival in this study [32]. Ibanez et al. screened ovarian tumors of different histology, with matched preoperative serum or plasma and peritoneal fluid samples for *RASSF1A* promoter methylation. They concluded for the first time that *RASSF1A* promoter methylation can be detected in cfDNA and represents an early event in ovarian carcinogenesis [33]. Other studies confirm the detection of methylated *RASSF1A* in plasma samples [34-36]. Bon Durant et al. compared *RASSF1A* promoter methylation between tumor and matched plasma, in 20 available sample pairs and observed 100% agreement. They also determined changes in *RASSF1A* methylation status during the course of treatment [34]. A phase II clinical trial has reported that demethylation of *RASSF1A* had a positive correlation with PFS indicating a possible role of *RASSF1A* promoter methylation in platinum resistance [37].

A large number of studies declare *RASSF1A* promoter methylation in the majority of human malignancies, including breast, endometrial and cervical cancer [23, 38]. Our group has shown the prognostic significance of *RASSF1A* promoter methylation in early stage breast cancer [39] and reported the frequent *RASSF1A* promoter methylation in cfDNA of operable gastric cancer patients [40]. Spitzwieser et al. investigated *RASSF1A* promoter methylation status in 17 breast cancer samples and their matched normal adjacent tissues using MS-HRMA and found high methylation frequencies in tumors and adjacent tissues, but no correlation between their methylation status [41]. A previous study also showed no significant concordance

between methylation changes in 56 breast tumor and their paired adjacent normal tissues [42]. In endometrioid adenocarcinoma, Arafa et al. reported that *RASSF1A* promoter methylation is methylated in endometrial cancer samples but also in a small group of adjacent normal endometrium tissues surrounding the tumor [43]. Evaluation of *RASSF1A* promoter methylation in matched samples of ovarian cancer patients has been very limited so far. There is only one study, including 3 tumors and their matched normal adjacent tissues, where all 3 adjacent tissues were found non-methylated [28]. Promoter methylation in adjacent morphologically tumor cell-free tissues reflects field cancerization, also called field effect. Field effect describes all the genetic and epigenetic abnormalities found in adjacent tissues that are defined as morphologically normal [44].

In the present study, we examined *RASSF1A* promoter methylation status in primary tumors, adjacent morphologically tumor cell-free tissues and corresponding plasma samples of patients with HGSC, using real-time MSP and MS-HRMA. The two assays showed almost perfect agreement when applied in the tumor samples of both groups, and substantial agreement in the adjacent tissues of group B. Two tumor samples from group A, 3 tumor and 6 adjacent samples from group B were found methylated with MS-HRMA and unmethylated with real-time MSP **(Table 1)**. These discrepant results probably are due to the slight differences in the promoter region assessed by each assay **(Figure 5)**. As shown in Figure 5, there is one extra CG in the forward MS-HRMA primer. When a sample is negative in real-time MSP, all CGs in MSP primers are unmethylated. But if the additional CG that is present only in the MS-HRMA primer is methylated in the sample, then the MS-HRMA result is expected to be positive. There were also 2 tumor samples from group A and 2 adjacent samples from group B where we observed methylation with real-time MSP, but not with MS-HRMA **(Table 1)**. This is potentially due to the higher sensitivity of the real-time MSP assay compared to MS-HRMA.

In adjacent morphologically tumor cell-free tissues of group B, we observed rather high methylation levels using both MS-HRMA (36.2%) and real-time MSP (29.3%). This indicates field cancerization (field effect) and potentially cancer progression. We found no significant difference between tumor and adjacent tissue methylation level and in three cases, the percentage of *RASSF1A* promoter methylation was higher in the adjacent tissue **(Figure 2)**. A potential explanation is the strong field effect that characterizes the cases studied, especially these three pairs.

According to our findings, *RASSF1A* promoter methylation is significantly correlated with OS when MS-HRMA is used (P=0.023), but no significant correlation is observed with real-time MSP (P=0.157). This fact gives an advantage to the MS-HRMA, although real-time MSP is a more sensitive assay. However, real-time MSP is preferable for methylation studies in plasma ctDNA, due to its higher sensitivity. In six samples, where the primary tumor was found unmethylated, the corresponding plasma samples were methylated. A potential explanation for this observation could be based on tumor heterogeneity; it is now clear that tissue biopsy represents a snapshot of tumor molecular profile, while cfDNA reflects the total genetic and epigenetic characteristics of a particular cancer. The effect of tumor heterogeneity in our results could be only shown if single cells analysis was performed. However we have not designed our study based on single cell analysis, this could be a nice idea for future studies. Moreover, cfDNA can originate not only from the primary tumor but from metastatic sites as well, from apoptotic and necrotic cells.

In conclusion, we performed a direct comparison study on *RASSF1A* promoter methylation in primary tumors, adjacent tissues and plasma samples in HGSC patients. We report for the first time that *RASSF1A* promoter is methylated in adjacent tissue surrounding the tumor in HGSC patients and that *RASSF1A* promoter methylation provides prognostic information since it is significantly correlated with OS. Our results indicate that the evaluation of *RASSF1A* methylation status in ovarian cancer has the potential to provide important prognostic information; however to verify this finding, more prospective studies should be performed. Taking into account that the primary tumor tissue is typically available only at primary diagnosis, it would be valuable to establish a non-invasive blood-based biomarker for stratifying response to platinum-based chemotherapy at primary diagnosis and for guiding individualized therapy decisions in the future.

MATERIALS AND METHODS

Clinical samples

Our study material consisted of two main groups of samples from patients with primary HGSC; a) group A that consists of 67 primary ovarian formalin fixed paraffin-embedded tissues (FFPEs) and b) group B that consists of 61 primary FFPEs, 58 available adjacent morphologically tumor cell-free tissues (FFPEs) and 59 available corresponding plasma samples (2mL). For the plasma sampling, two x 5ml ethylenediaminetetraacetic acid (EDTA) blood samples were collected at time point of diagnosis, before tumor surgery and before the application of therapeutic substances with an S-Monovette (Sarstedt AG & Co.). Blood was centrifuged at 1500g for 10min and the plasma supernatant was stored at -80°C until further usage. The available clinicopathological features for both groups are shown in **Table 5**. For the evaluation of the specificity of our assays, two groups of normal samples were recruited: a) a small group of 16 normal fallopian tube FFPEs that were obtained from women of the reproductive age group and b) a larger group of 51 plasma samples obtained from healthy women (2mL). All group A samples and the normal fallopian tube samples were obtained from the Pathology Department of IASO women's hospital, Athens, Greece. All group B samples were obtained from the Department of Pathology and the Department of Gynecology and Obstetrics, University Hospital of Essen, University of Duisburg-Essen, Germany. FFPE tissue blocks, retrieved from the Institute of Pathology and Neuropathology of the University Hospital of Essen, Germany were stained with Haematoxilin & Eosin and FFPE sections used for our assays were prepared and reviewed by a pathologist. We analyzed only samples with a tumor cell content of equal or more than 60%. All tissue samples were prepared under supervision of a pathologist. According to our pathologists, these are reasonable amounts of tumor tissue to study since 100% purity of tumor tissue can only be achieved in rare cases. All patients gave their informed written consent to participate in the study, which was approved by the Local Essen Research Ethics Committee (05/2856), and IASO women's hospital Ethics committee (Date: 05/2014). The OVCAR29 and IGROV1 ovarian cancer cell lines were used as positive controls in real-time MSP and MS-HRMA reactions for the detection of *RASSF1A* methylation.

DNA isolation from FFPEs and plasma samples

Genomic DNA (gDNA) was isolated from FFPEs using the QIAamp® DNA FFPE Tissue Kit 50 (Qiagen®, Germany) according to the manufacturer instructions. cfDNA from plasma (2mL) was extracted using the QIAamp® Circulating Nucleic Acid kit 50 (Qiagen®, Germany), according to the manufacturer's instructions. DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Sodium bisulfite conversion

1μg of gDNA and up to 0.5μg of cfDNA were chemically modified with sodium bisulfite (SB), in order to convert only the non-methylated cytosines to uracils, but not the methylated ones. SB conversion was performed with the EZ DNA Methylation-Gold™ Kit 200 (Zymo Research Corp., USA), according to the manufacturer's instructions. DNA was treated with the conversion reagent, incubated at 98˚C for 10min and at 64˚C for 2.5h. In each conversion reaction, dH_2O and gDNA from OVCAR29 or IGROV1 ovarian cancer cell lines were used as negative and positive control, respectively. The Universal Methylated Human DNA Standard (Zymo Research Corp., USA) was used as fully methylated control. To evaluate the quality of SB converted DNA in all our samples, we used unmethylated *BRMS1* primers that are specifically designed to detect unmethylated *BRMS1* sequences after SB conversion, as previously described [20]. Real-time PCR amplification occurred in all SB converted DNA samples. The SB converted DNA was stored at -70˚C until used.

Real-time methylation specific PCR (real-time MSP)

We performed real-time MSP for the detection of *RASSF1A* promoter methylation, using specific primers adapted from a previous study [25]. The position of the primers in the promoter sequence is shown in **Figure 5**. 1μl of SB converted DNA was added in the PCR reaction mix, which consisted of 1X PCR buffer (Promega, USA), 2mM $MgCl_2$ (Promega, USA), 0.2μM of each dNTP (Invitrogen, USA), 0.15μg/μL BSA (Sigma, Germany), 0.2μM of each primer (Integrated DNA Technologies, USA), 1X LC Green® (Idaho Technology, USA) and 0.05U/μL GoTaq® DNA polymerase (Promega, USA). dH_2O was added to a final volume of 10μL. Protocol conditions were: 1 cycle at 95˚C for 2min, followed by 45 cycles of: 95˚C for 10s, 65˚C for 15s and 72˚C for 20s, and a final cooling cycle at 40˚C for 30s. All real-time MSP reactions were performed in the LightCycler® 1.5 instrument (Roche Applied Science, Germany).

Methylation-sensitive high-resolution melting analysis (MS-HRMA)

For the semi-quantitative estimation of *RASSF1A* promoter methylation, we used specific primers adapted from a previous study [45]. The position of the primers in the promoter sequence is also shown in **Figure 5**. In this assay, methylation independent (MIP) primers allow for the equal amplification of both methylated and non-methylated target sequences. 1µl of SB converted DNA was added in the PCR reaction mix, which consisted of 1X PCR buffer (Promega, USA), 2.5mM $MgCl_2$ (Promega, USA), 0.2µM of each dNTP (Invitrogen, USA), 0.25µg/µL BSA (Sigma, Germany), 0.25µM of each primer (Integrated DNA Technologies, USA), 1X LC Green® (Idaho Technology, USA) and 0.05U/µL GoTaq® DNA polymerase (Promega, USA). dH_2O was added to a final volume of 10µL. The initial real-time PCR protocol conditions were: 1 cycle at 95˚C for 2min, followed by 50 cycles of: 95˚C for 10s, 63˚C for 15s and 72˚C for 20s, and a final cooling cycle at 40˚C for 30s. All reactions were performed in the LightCycler® 1.5 instrument (Roche Applied Science, Germany). After PCR amplification, MS-HRMA was performed in the HR-1 High Resolution Melter instrument (Idaho Technology, USA). Melting data acquisition began at 69˚C and ended at 95˚C, with a ramp rate of 0.30˚C/s. After melting transition, fluorescence data normalization was performed, so that the four (1-4) vertical cursors of the instrument software are positioned in new adjusted temperatures in the same numeric order, from left to right. At the ramp rate of 0.30˚C/s, a temperature range of 0.5˚C was set between each cursor pair. Finally, the derivative plots were displayed in order to compare each sample's melting peak with those of the controls and have the semi-quantitative estimation of the methylation level. The totally methylated and non-methylated products have a melting temperature (T_m) of 86˚C and 81˚C, respectively (**Figure 6a, b**).

Analytical validation of the assays

Analytical specificity: We first verified that unconverted gDNA was not detected. The Universal Methylated Human DNA Standard (100% methylated control) was used as the fully methylated positive control in both assays. We also checked *RASSF1A* promoter methylation status of OVCAR29 and IGROV1 cell lines. Both cell lines were found methylated by using the real-time MSP assay and their melting curves resembled to those of the 100% positive control, according to the MS-HRMA assay. We did not use human placental gDNA as a fully non-methylated control, as it is reported that *RASSF1A* promoter is methylated in placental DNA [46], a fact that we also verified by both real-time MSP and MS-HRMA. We have used normal fallopian

tube FFPE samples, as fully non-methylated controls, since unmethylated reference DNA from any tissue in which the target sequence does not show methylation, can be used as a source of unmethylated reference [47]. According to our results, both real-time MSP and MS-HRMA assays were highly specific, since *RASSF1A* promoter methylation was not detected at all both in the small group of fallopian tube FFPEs (0/16, 0%) and in the group of plasma samples from healthy women (0/51, 0%) (**Figure 6c, 6d**).

Analytical sensitivity: To estimate the analytical sensitivity of real-time MSP and MS-HRMA assays, we prepared synthetic standards by mixing one fully non-methylated DNA sample with the OVCAR29 cell line; we prepared serial dilutions: 0%, 0.1%, 1%, 10%, 30%, 50% and 100% for both assays. According to our results, real-time MSP assay detects down to 0.1% of *RASSF1A* promoter methylation in the presence of 99.9% non-methylated sequences (**Figure 7**), while MS-HRMA detects down to 1% of *RASSF1A* promoter methylation in the presence of 99% non-methylated sequence (**Figure 8**).

Statistical analysis

To estimate the agreement between the two assays in each sample group and the correlation of methylation status between subgroups of the group B, we calculated Pearson χ^2 and Cohen's Kappa coefficient. P values < 0.05 were considered statistically significant. The k values were interpreted according to the guidelines. The Kaplan-Meier method was used for the calculation of OS and PFS curves and log-rank test was performed for the comparisons. Cox regression analysis was also performed for the estimation of hazard ratio. All statistical analysis was performed by using the SPSS Windows version 22.0 (SPSS Inc., Chicago, IL).

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

TABLES

Tab.1: Comparison between real-time MSP and MS-HRMA for *RASSF1A* promoter methylation in primary tumors (n=128) and adjacent tissues (n=58)

Primary tumors: *RASSF1A* promoter methylation (group A, n=67)			
Real-time MSP	**MS-HRMA**		
	Unmethylated	Methylated	Total
Unmethylated	38	2	40
Methylated	2	25	27 (40.3%)
Total	40	27 (40.3%)	67
Agreement (methods)	63/67 (94.0%), P<0.001, Cohen's kappa=0.876		
Primary tumors: *RASSF1A* promoter methylation (group B, n=61)			
Real-time MSP	**MS-HRMA**		
	Unmethylated	Methylated	Total
Unmethylated	33	3	36
Methylated	0	25	25 (41.0%)
Total	33	28 (45.9%)	61
Agreement (methods)	58/61 (95.1%), P<0.001, Cohen's kappa=0.900		
Primary tumors: *RASSF1A* promoter methylation in both groups, (n=128)			
Real-time MSP	**MS-HRMA**		
	Unmethylated	Methylated	Total
Unmethylated	71	5	76
Methylated	2	50	52
Total	73	55	128
Agreement (methods)	121/128 (94.5%), P<0.001, Cohen's kappa=0.888		
Adjacent tissues: *RASSF1A* promoter methylation (group B, n=58)			
Real-time MSP	**MS-HRMA**		
	Unmethylated	Methylated	Total
Unmethylated	35	6	41
Methylated	2	15	17 (29.3%)
Total	37	21 (36.2%)	58
Agreement (methods)	50/58 (86.2%), P<0.001, Cohen's kappa=0.689		

Tab.2: *RASSF1A* promoter methylation in primary tumors, adjacent tissues and corresponding plasma samples using real-time MSP (n=53, triplets)

Primary tumors vs adjacent tissues: *RASSF1A* promoter methylation (n=53)			
Primary tumor	**Adjacent tissue**		
	Unmethylated	Methylated	Total
Unmethylated	30	2	32
Methylated	6	15	21
Total	36	17	53
Agreement	45/53 (84.9%), P<0.001, Cohen's kappa=0.674		
Primary tumors vs corresponding plasma: *RASSF1A* promoter methylation (n=53)			
Primary tumor	**Corresponding plasma**		
	Unmethylated	Methylated	Total
Unmethylated	26	6	32
Methylated	14	7	21
Total	40	13	53
Agreement	33/53 (62.3%), P=0.227, Cohen's kappa=0.156		

Tab.3: Correlation of *RASSF1A* methylation status of primary tumor samples with clinicopathological features of the patients (group B)

Clinicopathological characteristics	***RASSF1A* promoter methylation (primary tumors, n=47)**				
		real-time MSP		**MS-HRMA**	
	n[a]	**% methylation**	**P-value (χ^2 test)**	**% methylation**	**P-value (χ^2 test)**
Age					
≥ 64	24	9 (37.5)	0.908	11 (45.8)	0.642
< 64	23	9 (39.1)		9 (39.1)	
Tumor grade (G)					
G2	20	11 (55.0)	**0.043**	12 (60.0)	**0.037**
G3	27	7 (25.9)		8 (29.6)	
Regional lymph nodes (pN)					
N0	15	7 (46.7)	0.062 [b]	8 (53.3)	**0.040**
N1	20	3 (15.0)		4 (20.0)	

Distant metastasis (M)					
M0	41	16 (39.0)	1	18 (43.9)	1
M1	6	2 (33.3)		2 (33.3)	
Platinum resistance					
Positive	8	2 (25.0)	0.697[b]	4 (50.0)	0.454 [b]
Negative	34	12 (35.3)		12 (35.3)	
Tumor rest					
Positive	17	8 (47.1)	0.352	9 (52.9)	0.278
Negative	30	10 (33.3)		11 (36.7)	

a: in cases where the total number of patients is different this is due to non-available clinical information

b: Fisher's Exact Test

Tab.4: Univariate and multivariate Cox Regression analysis between OS and univariate Cox Regression analysis between PFS, *RASSF1A* methylation status of primary tumor samples and clinicopathological features of the patients (group B)

Univariate Cox Regression analysis (Dependent Variable: OS)				
	P-value	**HR (hazard ratio)**	**95.0% CI for HR**	
			Lower	**Upper**
***RASSF1A* methylation (real-time MSP)**	0.166	1.896	0.767	4.688
***RASSF1A* methylation (MS-HRMA)**	**0.030**	**2.761**	1.102	6.915
Age	0.844	0.913	0.370	2.254
Tumor grade (G)	0.744	0.860	0.348	2.126
Regional lymph nodes (pN)	0.432	0.640	0.210	1.948
Distant metastasis (M)	0.784	1.189	0.345	4.096
Platinum resistance	**0.019**	**3.752**	1.245	11.306
Tumor rest	0.758	0.859	0.326	2.263

Multivariate Cox Regression analysis (Dependent Variable: OS)				
***RASSF1A* methylation (MS-HRMA)**	0.253	1.818	0.653	5.064
Platinum resistance	**0.024**	**3.588**	1.185	10.863
Univariate Cox Regression analysis (Dependent Variable: PFS)				
	P-value	**HR (hazard ratio)**	**95.0% CI for HR**	
			Lower	**Upper**
***RASSF1A* methylation (real-time MSP)**	0.943	1.029	0.468	2.264
***RASSF1A* methylation (MS-HRMA)**	0.682	1.179	0.536	2.596
Age	0.827	1.093	0.494	2.416
Tumor grade (G)	0.401	1.405	0.636	3.106
Regional lymph nodes (pN)	0.599	0.773	0.296	2.018
Distant metastasis (M)	0.797	0.851	0.249	2.909
Platinum resistance	0.403	-	-	-
Tumor rest	0.597	1.244	0.554	2.792

Tab. 5: Available clinicopathological features of the patients

Clinicopathological characteristics	Group B (total n=64) n %	Group A (total n=67) n%
Histology		
Serous	64 (100)	67 (100)
Tumor grade (G)		
G1	2 (3.1)	-
G2	26 (40.6)	-
G3	36 (56.3)	67 (100)
FIGO stage		
I	1 (1.6)	13 (19.4)
II	2 (3.1)	38 (56.7)
III	39 (60.9)	12 (17.9)
IV	8 (12.5)	-
Unknown	14 (21.9)	4 (6.0)
Age	Median age=64	Median age=54
≥ median age	32 (50.0)	35 (52.2)
< median age	32 (50.0)	31 (46.3)
Unknown	-	1 (1.5)
Regional lymph nodes (pN)		
N0	18 (28.1)	
N1	29 (45.3)	
NX	4 (6.3)	
Unknown	13 (20.3)	
Tumor (pT)		
T1	6 (9.4)	
T2	6 (9.4)	
T3	52 (81.2)	
Distant metastasis (M)		
M0	55 (85.9)	
M1	8 (12.5)	
Unknown	1 (1.6)	
Platinum resistance		
Positive	10 (15.6)	
Negative	44 (68.8)	
Unknown	10 (15.6)	
Tumor rest		
Positive	25 (39.0)	

Negative	38 (59.4)	
Unknown	1 (1.6)	

FIGURE LEGENDS

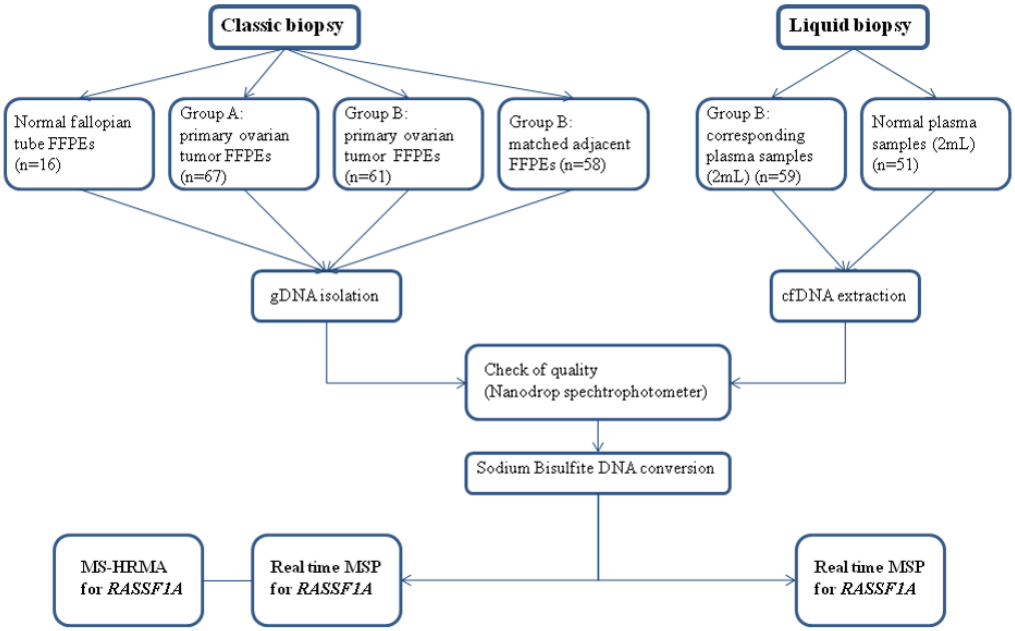

Fig.1: A schematic diagram of our study.

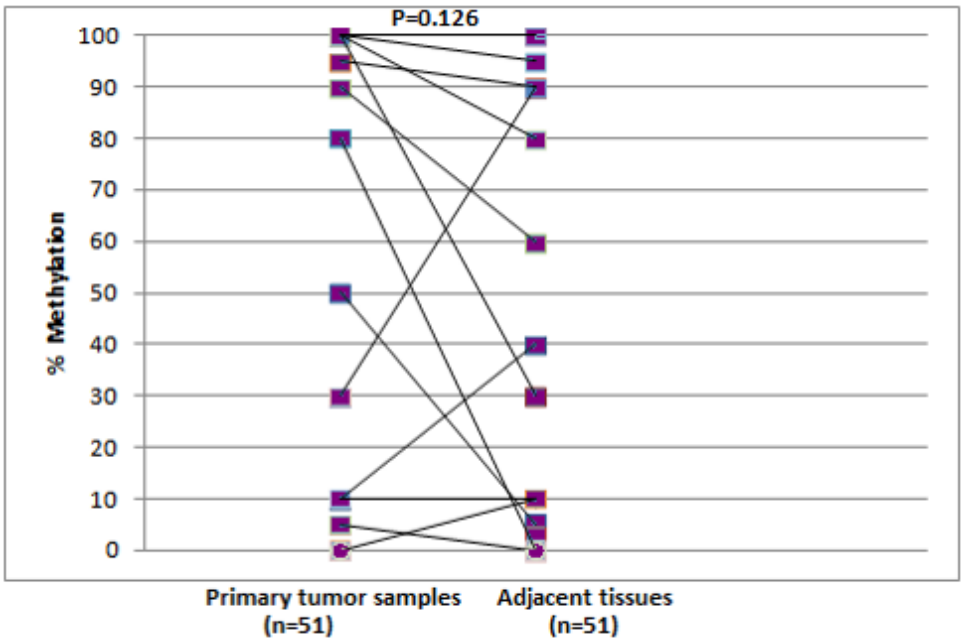

Fig.2: Comparison of *RASSF1A* promoter methylation levels in the paired primary tumor (n=51) and adjacent tissue (n=51) samples of group B, as estimated by MS-HRMA assay.

Fig.3: *RASSF1A* promoter methylation as evaluated both by real-time MSP and MS-HRMA, in group B: primary tumors, adjacent tissues and corresponding plasma samples (n=53). Red: positive sample (methylated), green: negative sample (unmethylated).

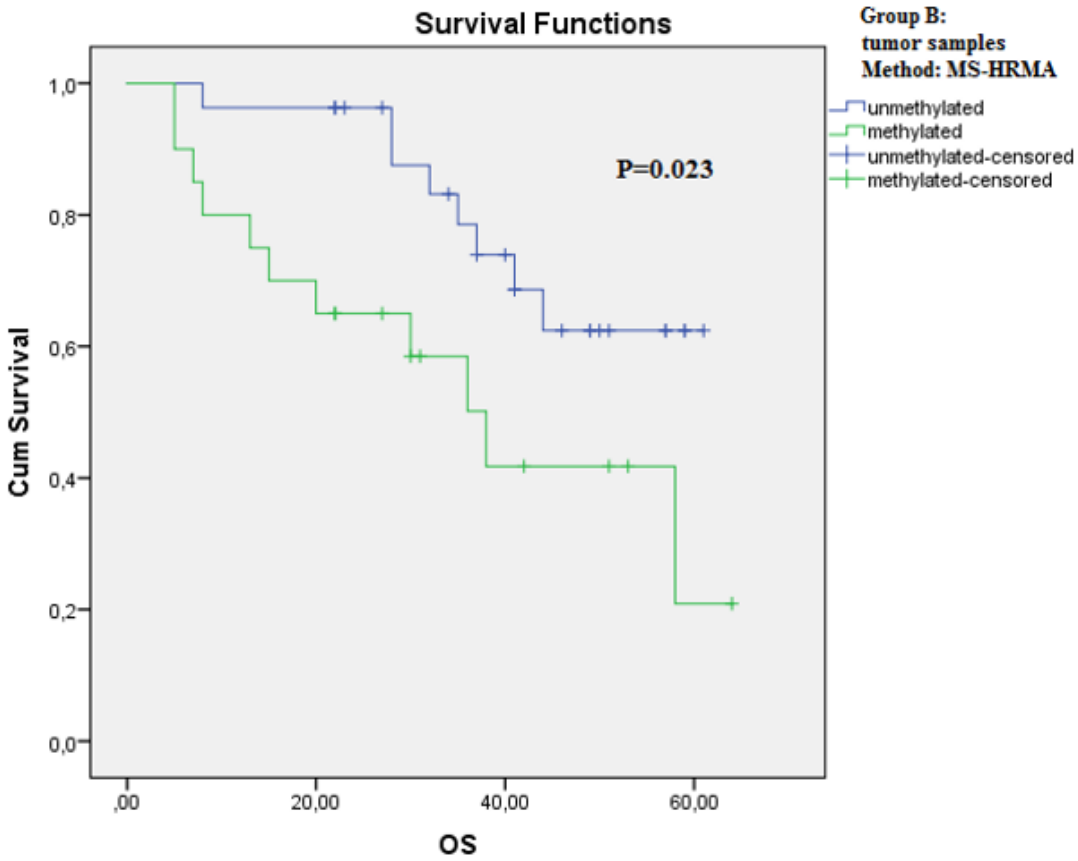

Fig. 4: Kaplan-Meier estimates of overall survival (OS) for patients of group B with (green) or without (blue) *RASSF1A* promoter methylation in tumor FFPEs using MS-HRMA (P=0.023).

CGTTGTTTTTTGGTTGTTTTTTTCGTTTTCGTAGGCGCGCGGGGTTATTATTTACGC
GCGTATTGTAGGTTTTTGCGTACGACGTTTTAGATGAAGTCGTTATAGAGGTCGTA
TTACGTGTGCGTGGCGGGTTTCGCGGGTTGGAAGCGGTGGTTACGGTTAGGGAT
TAGTTGTCGTGTGGGGTTGTACGCGGTGTTTCGCGCGATGCGTAGCGCGTTGGTA
CGTTTTAGTCGGGTGCGGTTTTTTTTAGCGCGTTTAGCGGGTGTTAGTTTTCGTAG
TTTAATGAGTTTAGGTTTTTTCGATATGGTTCGGTTGGGTTCGTGTTTCGTTGGTTT

Figure 5: Primer sequences and positions of real-time MSP and MS-HRMA assays for *RASSF1A* promoter methylation. The MSP primers are shown in blue underlined letters and the MS-HRMA primers are framed. The sequence is produced after SB conversion of gDNA. All CpGs are considered as methylated.

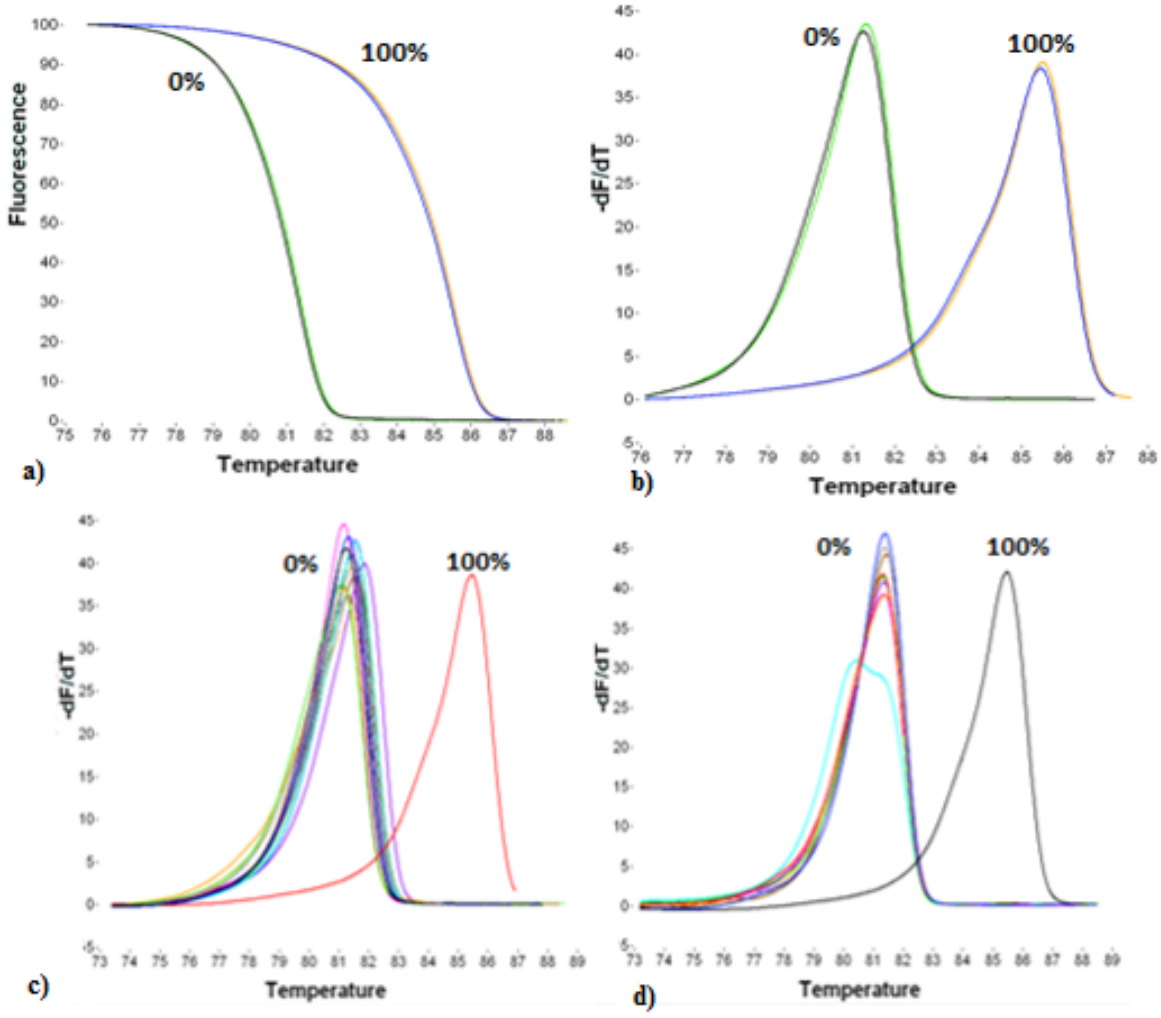

Fig. 6: Analytical specificity and reproducibility of the MS-HRMA assay:

a) Normalized melting curves of the fully non-methylated (0%) and the fully methylated (100%) control. b) Derivative plots of the 0% and 100% methylated controls. c) Derivative plots of the 16 normal fallopian tube FFPEs (0%). d) Derivative plots of normal plasma samples from healthy women (0%).

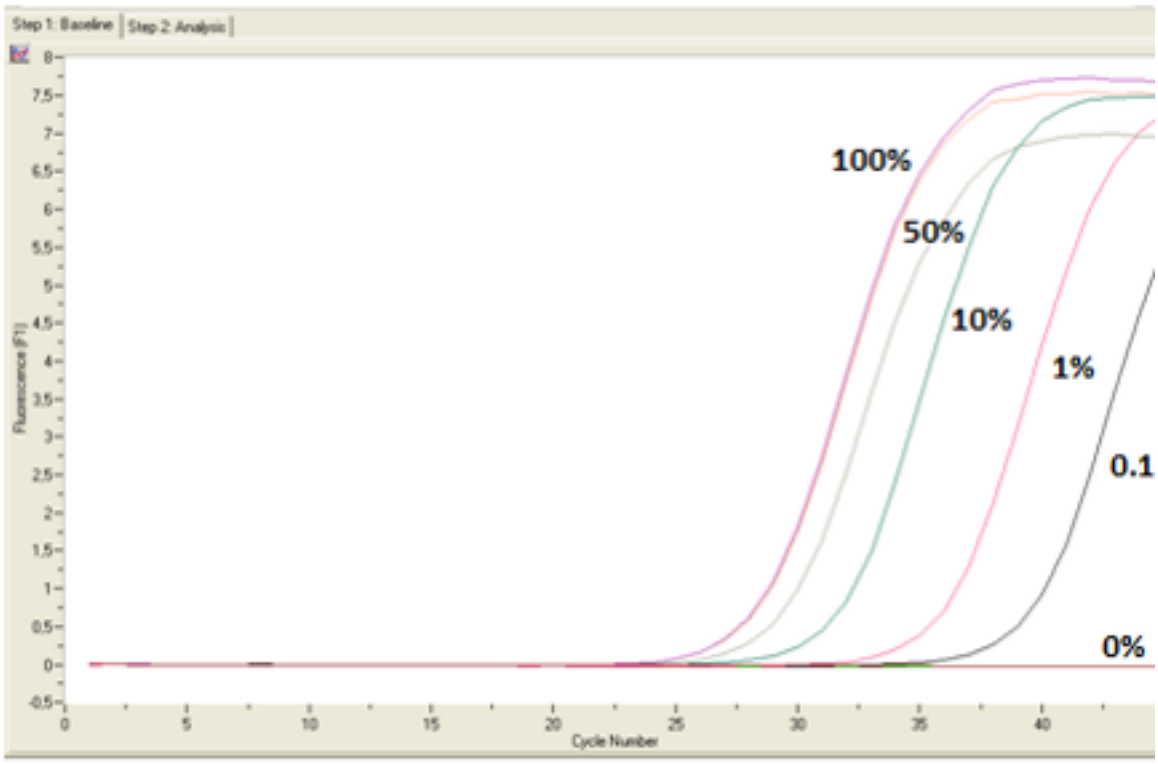

Fig.7: Analytical specificity and sensitivity of real-time MSP assay determined by the use of the dilutions (0%, 0.1%, 10%, 50%, 100%).

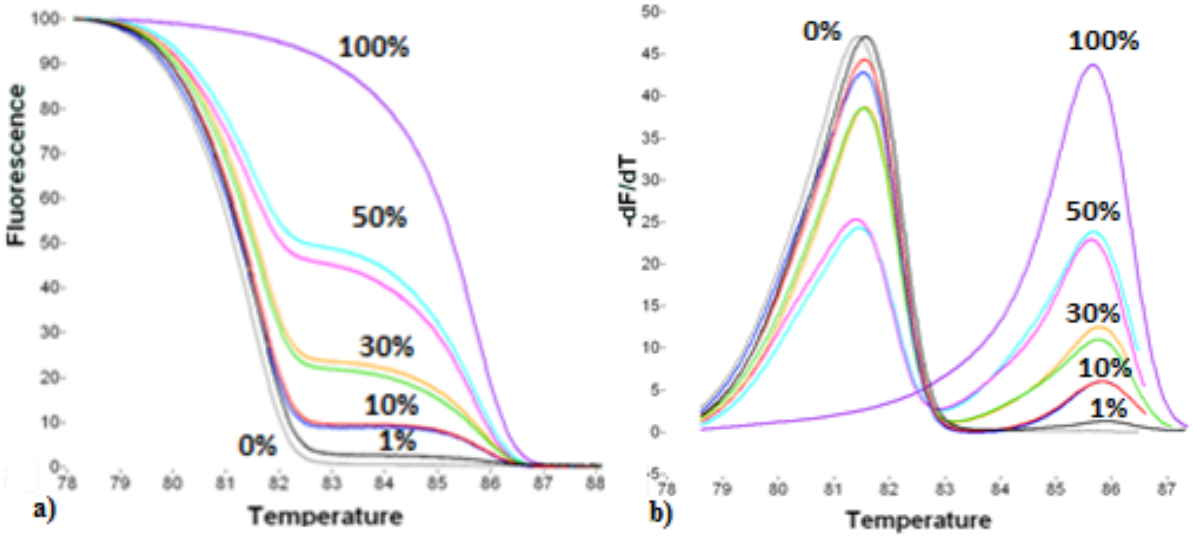

Fig.8: Analytical sensitivity and reproducibility of the MS-HRMA assay:

a) Normalized melting curves of the dilutions (0%, 1%, 10%, 30%, 50%, 100%).
b) Derivative plots of the same dilutions.

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4 Diskussion

Ziel dieser Dissertation war die Identifikation neuer Biomarkerkonzepte für das Ovarialkarzinom. Dies geschah zunächst auf Tumorstammzellebene mit dem Schwerpunkt der Analyse von persistierenden DTZ mit Stammzellcharakter im KM. Im zweiten Schritt wurden blutbasierte Strategien durchgeführt, um die Fragestellungen zu überprüfen, inwieweit die Präsenz und Persistenz von resistenten $ERCC1_{pos}$ ZTZ, ZTZ sowie ZTZ in EMT mit dem ungünstigen Verlauf der Erkrankung assoziiert sind. Als weitere, nicht zelluläre, „Liquid Biopsy" wurden zirkulierende DNA und microRNAs als neue bzw. ergänzende Marker evaluiert.

Zielsetzung 1:

DTZ mit Stammzelleigenschaften

Die Ergebnisse zu dieser Zielsetzung wurden in Oncotarget publiziert. *Analysis of disseminated tumor cells before and after platinum based chemotherapy in primary ovarian cancer. Do stem cells predict prognosis?*
Issam Chebouti,Christina Blassl, Pauline Wimberger, Hans Neubauer, Tanja Fehm, Rainer Kimmig, Sabine Kasimir-Bauer. Oncotarget. 2016. doi:3;7(18):26454-64.

Die prognostische Bedeutung der DTZ zu Beginn der Erkrankung im Hinblick auf das PFS und OS wurde bereits von verschiedenen Arbeitsgruppen bestätigt (Wimberger et al., 2007; Banys et al., 2009; Fehm et al., 2013; und Cui et al., 2015). Untersuchungen an DTZ nach platinbasierter Chemotherapie wurden bisher von anderen Gruppen nicht durchgeführt.

Unter Verwendung der Standardmethode zum Nachweis Ck_{pos} DTZ im KM (siehe Abb.1.12) wurden in der hier untersuchten Kohorte von 79 Patientinnen, bei 42% vor und bei 41% nach Therapie DTZ im KM detektiert, was die Verteilung der DTZ in bereits publizierten Studien der Arbeitsgruppe widerspiegelte. DTZ vor Therapie assoziierten signifikant mit einem verkürzten OS und die Patientinnen, die vor Therapie DTZ_{neg}, aber nach Therapie DTZ_{pos} waren, zeigten ein signifikant verkürztes PFS. Patientinnen mit persistierenden DTZ (16%) zeigten einen Trend zum schlechteren PFS/OS, der jedoch nicht signifikant war.

Bisher wurden Tumorstammzellen nur im ovariellen Tumorgewebe analysiert, wobei die Expression von LIN-28, SOX-2 und OCT-4 als wesentliche Marker beschrieben wurden (Peng et al., 2010; Bareis et al., 2013 und Pham et al., 2013). Für die Analyse der DTZ im KM wurde im ersten Promotionsjahr, in Zusammenarbeit mit Frau Christina

Blassl aus der Frauenklinik Düsseldorf, eine Vierfach-Immunfluoreszenzfärbung etabliert. Hierzu wurde die ovarielle Zelllinie OVCAR-3 sowie die Leukämiezelllinie Kasumi-1 verwendet. Die Methode wurde dann auf KM-Präparate der Patientinnen vor und nach platinbasierter Therapie angewendet. Nachgewiesen wurden die Marker LIN-28 und SOX-2 als Stammzellmarker für das Ovarialkarzinom, der Zytokeratinmarker C11, der die Zytokeratine 4,5,6,8,10,13 und 18 detektiert, sowie CD34 als hämatopoeitischer Stammzellmarker und CD45 als Leukozytenmarker zum Ausschluss falsch-positiver Zellen. Die Untersuchungen am KM von Patientinnen konnten nicht an den bereits mit der Standard-Methode untersuchten Präparaten durchgeführt werden, da die Neufuchsinfärbung weitere Färbungen nicht erlaubt. Somit wurde auf Reservepräparate der Patientinnen zurückgegriffen. Aufgrund der geringen Anzahl an Reserveobjektträgern wurden nur die Stammzellmarker LIN-28 und SOX-2 untersucht. Die Tab. 4.1 zeigt zur Veranschaulichung der Ergebnisse eine Übersicht der analysierten 10 Patientinnen. Es wurden drei Gruppen gebildet, basierend auf den mit der Standardmethode detektieren CK_{pos} DTZ vor und nach Therapie (Spalten grau unterlegt). Gruppe 1 beschreibt sechs Patientinnen (1,3,4,7,8,10), die vor Therapie keine DTZ, nach Therapie jedoch Zellen aufwiesen. Die zweite Gruppe stellt die Patientinnen dar, die vor Therapie DTZ_{pos} waren, nach Therapie aber keine Zellen aufwiesen (2, 5). Gruppe 3 umfasst die Patientinnen mit persistierenden DTZ (6,9). Betrachtet wurden zunächst die Patientinnen (1,3,4,6, 7,8,9,10), die nach Therapie mindestens fünf Ck_{pos}-Zellen (DTZ) aufwiesen, sodass die Wahrscheinlichkeit groß war, auch auf den Reservepräparaten Zellen zu detektieren. Bei 9/10 Patientinnen wurden Ck_{pos}/Lin-28_{pos} Zellen und bei 7/10 Patientinnen Ck_{neg}/Lin-28_{pos} Zellen gefunden. Ähnliche Ergebnisse wurden für SOX-2 nachgewiesen. Bei sechs dieser Patientinnen (1,3,4,7,8,10) zeigten die korrespondierenden KM-Präparate vor Therapie, die mit der Standardmethode Ck_{neg} (DTZ_{neg}) waren, interessanterweise die Präsenz einzelner LIN-28_{pos} und/oder SOX-2_{pos} Zellen. Ähnliches galt für Patientinnen, die nach Therapie mit der Standardmethode als Ck_{neg} (DTZ_{neg}) charakterisiert wurden (2,5). Bei Patientinnen mit persistierenden DTZ (6,9) wurden sowohl vor, als auch nach Therapie Zellen mit Stammzellcharakter nachgewiesen. Diese Resultate könnten das signifikant verkürzte PFS von Patientinnen erklären, die anfänglich vor Therapie keine DTZ hatten und nach Therapie DTZ aufwiesen. Auch bei Patientinnen, die nach Therapie keine Ck_{pos} DTZ, also epitheliale Zellen aufwiesen, konnten vereinzelt LIN-28_{pos}/SOX-2_{pos} Zellen detektiert werden. Inwieweit diese Zellen ebenfalls die Prognose beeinflussen, konnte

aufgrund der geringen Anzahl an Patientinnen nicht evaluiert werden. Ein weiterer, interessanter Befund unserer Untersuchungen war die Detektion zweier unterschiedlicher Zellpopulationen (Abb.4.1). Einerseits detektierten wir Zellen, die **Ck_{pos}**/SOX-2_{pos} (LIN-28_{pos}), andererseits **Ck_{neg}**/SOX-2_{pos} (LIN-28_{pos}) waren (Abb.4.1).

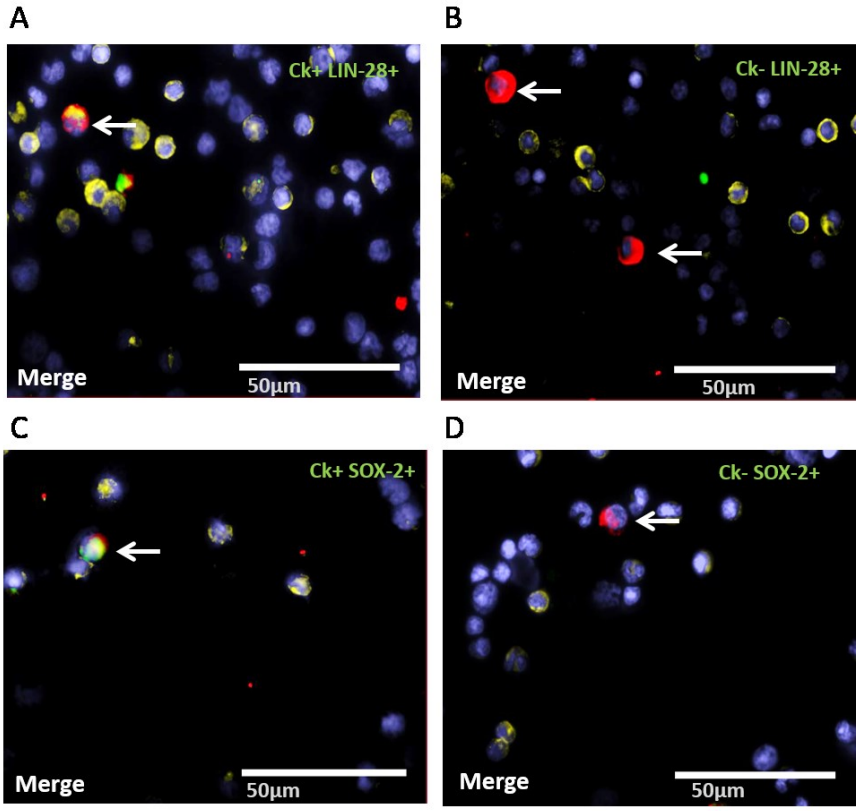


Abb.4.1 Exemplarische Beispiele der detektierten DTZ mit Stammzellcharakter
Durchführung der Experimente erfolgten in Kooperation mit Frau Blassl aus der Frauenklinik Düsseldorf.
(Chebouti et al., 2016)

A: Merge Ck_{pos} LIN-28_{pos}; B: Merge Ck_{neg} Lin-28_{pos}; C: Merge Ck_{pos} SOX-2_{pos}; Ck_{neg} SOX-2_{pos} (Alle Zellen $DAPI_{pos}$, $CD34_{neg}$, $CD45_{neg}$)

Das legt die Vermutung nahe, dass die Expression der Stammzellmarker LIN-28 und/oder SOX-2 nach Therapie für ein schlechtes OS verantwortlich sein könnten. Des Weiteren vermuten wir, dass es sich bei der Zellpopulation Ck_{neg}/SOX-2_{pos} (LIN-28_{pos}) möglicherweise um Zellen in EMT handelt, während die Zellpopulation Ck_{pos}/SOX-2_{pos} (LIN-28_{pos}) keine Umwandlung durchlaufen oder den mesenchymalen Charakter verloren hat und wieder epithelial geworden ist. Es ist bekannt, dass SOX-2, das an die SRC-Kinase bindet, einer Nicht-Rezeptor Tyrosin-Kinase, die Zellmigration, Invasion und Adhäsion von serösen Karzinomzellen erhöht (Wang et al., 2014). Die Hemmung, entweder von LIN-28 oder OCT-4- Expression, verringert die Zellviabilität.

Tab. 4.1 Verteilung der DTZ und LIN-28 sowie SOX-2-positiven Zellen bevor und nach Therapie

			bevor Therapie				nach Therapie			
Patienten	**DTZ_{pos} bevor Therapie (A45-B/B3)**	**DTZ_{pos} nach Therapie (AF45-B/B3)**	**Ck_{pos}/LIN-28_{pos}**	**Ck_{neg}/LIN 28_{pos}**	**Ck_{pos}/SOX-2_{pos}**	**Ck_{neg}/SOX-2_{pos}**	**Ck_{pos}/LIN-28_{pos}**	**Ck_{neg}/LIN-28_{pos}**	**Ck_{pos}/SOX-2_{pos}**	**Ck_{neg}/SOX-2_{pos}**
1	0	14	25	5	24	2	4	0	3	4
2	37	0	2	0	4	1	0	1	2	0
3	0	11	8	2	5	1	2	0	0	1
4	0	15	2	1	1	1	2	1	kov	kov
5	6	0	1	1	2	1	1	0	2	1
6	28	18	kov	kov	2	3	3	7	4	11
7	0	5	kov	kov	kov	kov	1	3	0	2
8	0	100	kov	kov	kov	kov	5	9	1	2
9	1	35	kov	kov	kov	kov	2	1	kov	kov
10	0	10	kov	kov	kov	kov	3	11	2	9

***kov= keine Objektträger vorhanden**

Die kombinierte Repression von LIN-28 und OCT-4 führt zu einer synergetischen Inhibition beim Wachstum der Tumorzellen und beim Überleben von ovariellen Tumorzelllinien (Bapat et al., 2005). Die Expression von SOX-2 wurde mittels Immunhistochemie sowohl an normalem, ovariellen epithelialen, serösen und muzinösen Zystadenomen, als auch an Zystadenomkarzinomen untersucht (Ye et al., 2011). Eine Überexpression von LIN-28 wurde in verschiedenen epithelialen Tumoren, darunter Mamma-, Bronchial-, Kolon- und Ovarialkarzinomen, detektiert (Viswanathan et al., 2009). Bei Patientinnen mit Ovarialkarzinom, die eine taxanbasierte Chemotherapie erhielten, korrelierte die Expression von SOX-2 signifikant mit der Chemoresistenz und einem verkürztem PFS, während eine fehlende taxanbasierte Chemotherapie, keine Signifikanz zeigte (Du et al., 2015). Da die Patientinnen in unserer Studie auch eine kombinierte Therapie mit Paclitaxel und Carboplatin erhalten haben, könnte es möglich sein, dass die SOX-2 Expression an DTZ mit einer Chemotherapie-Resistenz assoziiert ist. Dennoch bleiben Mechanismen, die für eine Chemotherapie-Resistenz beim Ovarialkarzinom verantwortlich sind, noch ungeklärt.

Zielsetzung 2:

Untersuchungen an ZTZ

Die zu diesem Thema erhobenen Daten wurden in Oncotarget publizert.

EMT-like circulating tumor cells in ovarian cancer patients are enriched by platinum-based chemotherapy.
Issam Chebouti, Sabine Kasimir-Bauer, Paul Buderath, Pauline Wimberger, Siggi Hauch, Rainer Kimmig, Jan Dominik Kuhlmann. Oncotarget 2017, in press.

ERCC1-expressing circulating tumor cells as a potential diagnostic tool for monitoring response to platinum-based chemotherapy and for predicting post-therapeutic outcome of ovarian cancer.
Issam Chebouti, Jan Dominik Kuhlmann, Paul Buderath, Stephan Weber, Pauline Wimberger, Yvonne Bokeloh, Siggi Hauch, Rainer Kimmig, Sabine Kasimir-Bauer. Oncotarget 2016, doi:18632/oncotarget.13286.

Da die Analyse der DTZ sehr invasiv ist, ungeeignet als Methode zur Beurteilung des Krankheitsverlaufs, und zudem momentan keine therapeutische Konsequenz beinhaltet, lehnen die Patientinnen zum größten Teil eine KM-Punktion nach Therapie ab. Deshalb sind blutbasierte Studien von immer größerer Bedeutung. Die Arbeitsgruppe um Frau Professorin Kasimir-Bauer konnte schon relevante Beiträge zur Etablierung neuer, blutbasierter Biomarkerkonzepte für das Ovarialkarzinom publizieren und die Hypothese unterstützen, dass Patientinnenblut im Sinne einer „Real-Time-Liquid-Biopsy“ genutzt werden könnte. Im Hinblick auf ZTZ wurde die

schlechte Prognose der Patientinnen hinsichtlich des PFS und OS schon belegt (Wimberger et al., 2007; Aktas et al., 2011; Kuhlmann et al., 2014).

Im Rahmen dieser Doktorarbeit wurde untersucht, inwieweit auch ZTZ in EMT in der Population der ZTZ präsent sind. Zu dieser Fragestellung gibt es momentan in der Literatur keine Daten. Aufgrund der in der bereits publizierten multivariablen Analyse zur prognostischen Bedeutung der $ERCC1_{pos}$ ZTZ vor Therapie (Kuhlmann et al., 2014), ergab sich die Frage, ob diese Zellen nach Therapie präsent sind bzw. persistieren und den schlechten Verlauf der Erkrankung bei einigen Patientinnen erklären.

A) ZTZ in EMT

Diese Analyse wurde an Blutproben von insgesamt 91 Patientinnen vor und 31 nach platinbasierter Chemotherapie durchgeführt. Unsere wesentlichen Ergebnisse zeigten, dass der mesenchymale Phänotyp sowohl vor als auch nach Therapie dominierte und sogar um 20% nach Therapie zunahm (Abb.4.2). Bei genauer Analyse der Markerexpression zeigte sich, dass sich unter Therapie ein ZTZ-Phänotyp (PI3Kα+Twist) entwickelte, der vor Therapie nicht präsent war (Abb.4.3).

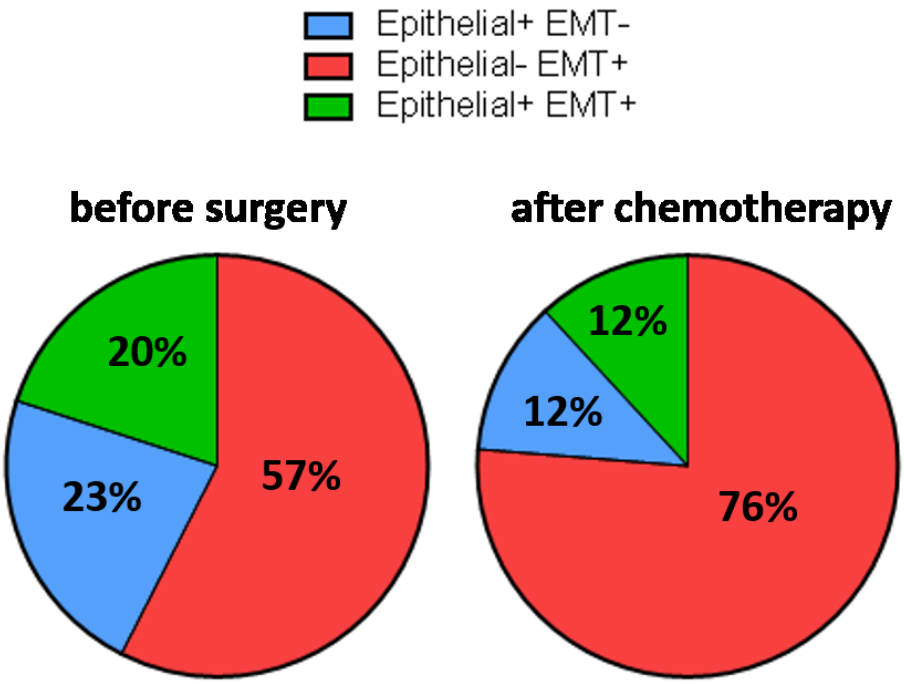


Abb.4.2 Schematische Darstellung der Distribution der ZTZ-Phänotypen vor und nach der Chemotherapie (Chebouti et al., 2017)

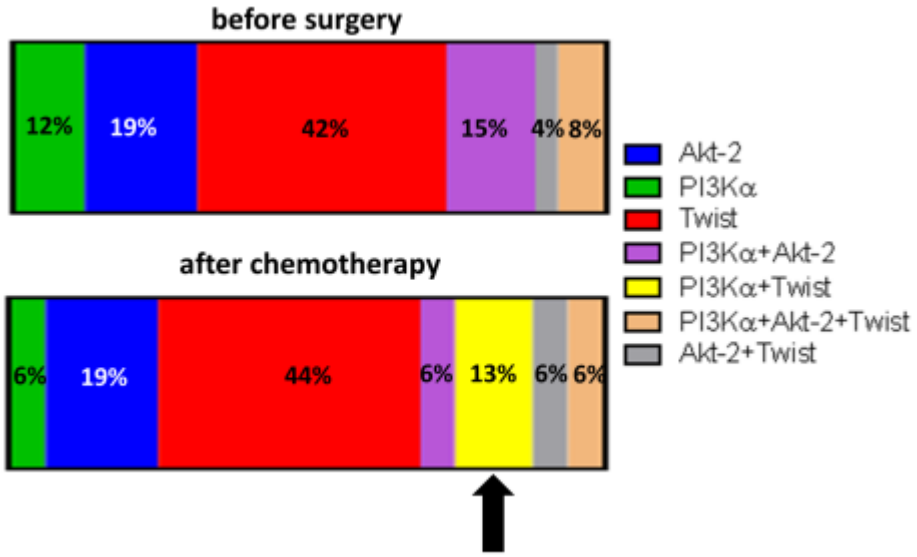


Abb.4.3 ZTZ Phänotypen vor und nach Therapie im Vergleich (Chebouti et al., 2017)

Die Präsenz PI3Kα+EMT-ähnlicher ZTZ in Kombination mit epithelialen ZTZ korrelierte signifikant mit einem verkürzten OS. FIGO I-III-Patientinnen mit Resttumoren nach der Operation waren eher positiv für EMT-ähnliche ZTZ nach Chemotherapie. In der letzteren Gruppe korrelierten epitheliale ZTZ signifikant mit einem verkürzten PFS und OS, unterstützt durch die zusätzliche Einbeziehung von $PI3K\alpha_{pos}$ ZTZ.

Wir verwendeten in unserer Studie ausdrücklich den Begriff EMT-ähnliche ZTZ und die strikte Vermeidung von anderen beschreibenden Begriffen, die implizieren könnten, dass diese ZTZ bereits einen vollständig mesenchymalen Zustand erwarben. Da die immunmagnetische Anreicherung von ZTZ in unserem Assay auf den epithelialen Oberflächenepitopen MUC und EpCAM basiert, kann dieser Assay nicht vollständig mesenchymale ZTZ nachweisen, da diese ihre epithelilalen Oberflächenproteine vollständig herunterreguliert haben. Somit exprimieren EMT-ähnliche ZTZ, die hier ausgewählt und charakterisiert sind, die Marker EpCAM und MUC-1 auf ihrer Oberfläche, was eine immunmagnetische Selektion erlaubt. Jedoch erlaubt diese Methode keine Detektion der Expression von EpCAM oder MUC-1 auf Transkriptionsebene oberhalb der Nachweisgrenze unseres Assays. Auf den ersten Blick lassen sich Diskrepanzen zwischen Protein- und Transkript-Expressionsprofilen einer Zelle durch posttranskriptionelle Modifikation der mRNA oder Unterschiede in der Halbwertszeit zwischen mRNA und ihren entsprechenden Proteinen erklären (Greenbaum et al., 2003; Nie et al., 2006; Pascal et al., 2008). Da diese ZTZ auch AKT-2, PI3Kα oder Twist co-exprimieren, beschreiben wir die Momentaufnahme von „semi-mesenchymalen“ ZTZ (Bednarz-Knoll et al., 2012). Semi-mesenchymale ZTZ

könnten entweder auf ihrem Weg zu einem letztlich mesenchymalen Phänotyp (EMT) oder auf ihrem Weg zurück zu einem epithelilalen Phänotyp (MET) sein. Alternativ könnten sie in diesem Zwischenzustand verharren. Obwohl die Biologie von semi-mesenchymalen ZTZ weitgehend unbekannt ist, können wir annehmen, dass insbesondere ein semi-mesenchymaler Zustand einen aggressiven ZTZ-Phänotyp mit hohem Plastizitätsgrad wiederspiegelt, der die Anpassung von ZTZ an die umgebenden Reize während der Streuung erleichtert. Dies steht im Einklang mit der Hypothese, dass EMT (und der Reversion-MET) ein hochdynamischer Prozess ist und verschiedene kontinuierliche Phänotypen beschreibt, anstatt einen dichotomen Wechsel zwischen epithelialen und mesenchymalen Zuständen. Insbesondere beeinflussen diese phänotypischen Veränderungen direkt die Wiederfindungsrate von ZTZ-Nachweis-Assays, die auf epithelialen Selektionsmarkern basieren (Bednarz-Knoll et al., 2012). Jedoch wird unsere Annahme von kontinuierlichen ZTZ-Phänotypen beim Ovarialkarzinom nicht notwendigerweise durch die Tatsache unterstützt, dass wir einen Trend für einen gegenseitigen Ausschluss zwischen Patientinnen mit nur epithelialen ZTZ und denen mit nur semi-mesenchymalen ZTZ beobachteten. Dies ist ein sehr interessanter Befund und kann zeigen, dass es auch eine Untergruppe von Ovarialkarzinompatientinnen mit ausschließlich epithelialen ZTZ gibt, ohne Verschiebungen in Richtung EMT. Die klinische Relevanz dieses Befundes erfordert jedoch weitere Untersuchungen. Mit Hilfe unseres EMT-assoziierten Markersets haben wir ein heterogenes Spektrum von EMT-assoziierten ZTZ-Phänotypen aufgezeigt, was auf eine ZTZ-Heterogenität bei Ovarialkarzinomen hinweist, die bereits für ZTZ in einer Vielzahl anderer Tumorentitäten, wie dem Prostata- oder Mammakarzinom, publiziert wurde (Massard et al., 2016; Shaw et al., 2016). Zu bedenken ist, dass wir mit unserem Assay nicht unterscheiden können, ob eine Expression mehrerer Marker-Transkripte von einer ZTZ stammt, die diese Marker in der gleichen Zelle exprimieren, oder aus separaten semi-mesenchymalen ZTZ-Populationen von angereicherten ZTZ. Wir gehen daher von einer klonalen Selektion von ZTZ mit aktivierten PI3Kα- und Twist-assoziierten Signalwegen aus, die therapeutisch refraktär und für das posttherapeutische Rezidiv des Ovarialkarzinoms verantwortlich sein könnten. Diese Hypothese wird durch unsere bisherigen Studien an Mammakarzinompatientinnen stark unterstützt, in denen bereits gezeigt wurde, dass nach der neoadjuvanten Chemotherapie bevorzugt semi-mesenchymale und möglicherweise platinresistente (ERCC1-exprimierende) ZTZ verbleiben (Kasimir-Bauer et al., 2016). Darüber hinaus berichtete eine weitere unabhängige

Schlüsselveröffentlichung über dynamische Veränderungen in der epithelialen und mesenchymalen Zusammensetzung von Mammakarzinom-ZTZ als Reaktion auf die Therapie. In dieser Studie wurde das klinische Ansprechen von einer Veränderung zu überwiegend epithelialen ZTZ begleitet, während progressive Erkrankungen mit dem Anstieg der mesenchymalen ZTZ-Phänotypen korrelierten (Yu et al., 2013). In vitro-Experimente zeigten, dass eine Cisplatinbehandlung von Ovarialkarzinomzellen, Zellen mit EMT-ähnlichen Merkmalen generieren (Latifi et al., 2011). Daher nehmen wir an, dass PI3Kα$_{pos}$ und Twist$_{pos}$ ZTZ die Tumorentwicklung als Reaktion auf eine platinbasierte Chemotherapie widerspiegeln könnten. Interessanterweise deutet dies auch auf einen Zusammenhang mit neueren Studien zur genomischen Tumorentwicklung hin, die über eine Zunahme der Aktivierung der PI3Kα-Mutation unter zellfreier Tumor-DNA von Mammakarzinompatientinnen nach Paclitaxel-behandlung berichteten (Murtaza et al., 2013). In diesem Zusammenhang könnten unsere Befunde mehrere diagnostische oder therapeutische Auswirkungen haben, da PI3Kα und Twist funktionell an Signalwegen beteiligt sind, die das Überleben von Tumoren oder die Platinresistenz kontrollieren (Kolasa et al., 2009, Nuti et al., 2014). Weiterhin wird SOX-2 in Zusammenhang mit der Chemoresistenz diskutiert. So zeigten Patientinnen mit taxanbasierter Chemotherapie und SOX-2-Expression im Gewebe eine Chemotherapieresistenz sowie ein verkürztes PFS (Du et al., 2015). Hier zeigt sich ein Zusammenhang mit den zuvor beschriebenen Daten an DTZ.

B) ZTZ mit Resistenzcharakter

Ungefähr 15-20% der Patientinnen mit Ovarialkarzinom, die eine platinbasierte Chemotherapie erhalten, zeigen eine Platinresistenz, was das größte Problem dieser Erkrankung darstellt. Die Expression von ERCC1 wird als möglicher Biomarker für Platinresistenz diskutiert, jedoch waren immunhistochemische Bestimmungen beim Ovarialkarzinom und anderen soliden Tumoren wenig informativ (Friboulet et al., 2013). Auch die Arbeitsgruppe der Frauenklinik konnte dies bestätigen, jedoch war die Präsenz von ERCC1$_{pos}$ ZTZ zu Beginn der Erkrankung ein unabhängiger Prädiktor für das PFS, OS und für die Platinresistenz (Kuhlmann et al., 2014).

Im Rahmen dieser Dissertation wurde an dieser Thematik weitergearbeitet. Im Vordergrund stand die Frage, ob ERCC1$_{pos}$ ZTZ, die schon zu Beginn der Erkrankung präsent waren, auch nach Therapie persistieren oder ob sich diese Zellpopulation auch unter Therapie entwickeln kann. In einer Kohorte von 65 Patientinnen zeigten 15% der Patientinnen vor und 12% nach Therapie ERCC1$_{pos}$ ZTZ. Die Abb.4.4 fasst die

detektierten ZTZ-Phänotypen zusammen und zeigt ihre relativen Proportionen innerhalb den untersuchten Ovarialkarzinompatientinnen. Als AdnaTest$_{pos}$ wurden die Patientinnen bezeichnet, die mindestens für eines der Transkripte EpCAM, MUC-1 oder CA-125 positiv waren. Außerdem wurde über eine zusätzliche „single-plex“ PCR die Expression von ERCC1-Transkripten analysiert. Vor Therapie wurde bei 8% der Patientinnen ausschließlich eine AdnaTest-Positivität beobachtet. In 17% der Fälle wurden exklusiv ERCC1$_{pos}$ ZTZ detektiert und bei 15% der Patientinnen beobachteten wir eine Positivität für AdnaTest und ERCC1 (Abb.4.4A). Anschließend waren wir daran interessiert, wie die Bewertung von ERCC1$_{pos}$-Patientinnen die Gesamtnachweisrate von ZTZ bei Ovarialkarzinompatientinnen beeinflusst. Daher wurde die Gesamt-ZTZ-Detektionsrate mit der Präsenz von ERCC1-Transkripten als zusätzliches, alternatives Kriterium untersucht (Abb. 4.4B): Vor Therapie wurde eine ZTZ-Detektionsrate von 23% beobachtet, bestehend aus Patientinnen mit nur AdnaTest Positivität UND ERCC1$_{neg}$ ODER ERCC1$_{pos}$ Patientinnen. Die Detektionsrate wurde bis zu 40% deutlich erhöht, wenn ERCC1 als weiterer, alternativer Marker für die ZTZ-Positivität (AdnaTest$_{pos}$ ODER ERCC1$_{pos}$) betrachtet wurde. Die Subgruppe AdnaTest$_{neg}$ UND ERCC1$_{pos}$ zeigte eine Gesamtnachweisrate von 17%. Schließlich sank nach einer strengeren Definition der kombinierten Positivität (AdnaTest$_{pos}$ UND ERCC1$_{pos}$) die Gesamtdetektion auf 15%. Nach der platinbasierten Chemotherapie war der Anteil der ZTZ-Subtypen und deren Gesamtnachweisraten unter den oben genannten ZTZ-Definitionskriterien mit denen, die vor der Therapie gefunden wurden, vergleichbar (Abb.4.4 C + D). Außerdem korrelierte die Präsenz von ERCC1$_{pos}$ ZTZ mit einem verkürztem PFS und OS sowie mit der Platinresistenz. Diese Analyse könnte für die Vorhersage von posttherapeutischen Ergebnissen und für die Überwachung der platinbasierten Chemotherapie nützlich sein. Durch die zusätzliche starke prognostische Bedeutung der Marker MUC-1, EpCAM und CA-125 (Aktas et al., 2011 und Kuhlmann et al., 2014) und unter Berücksichtigung, dass das statistische Signifikanzniveau leicht rückgängig war, wenn nur ERCC1 Transkripte beurteilt wurden, scheint eine Bedingung, die eine ERCC1-Positivität und mindestens einen AdnaTest Marker umfasst, im Sinne eines blutbasierten prognostischen Marker am günstigsten zu sein. Bisher sind funktionelle Merkmale von ERCC1-exprimierenden ZTZ im Blut von Ovarialkarzinompatientinnen unbekannt. Da unsere Studie ausschließlich aus der „Biomarkerperspektive“ durchgeführt wurde, können wir nur annehmen, dass ERCC1-(über)exprimierende ZTZ im Blut durch eine erweiterte, oder bereits vorhandene oder neu erworbene Fähigkeit, charakterisiert werden können, um

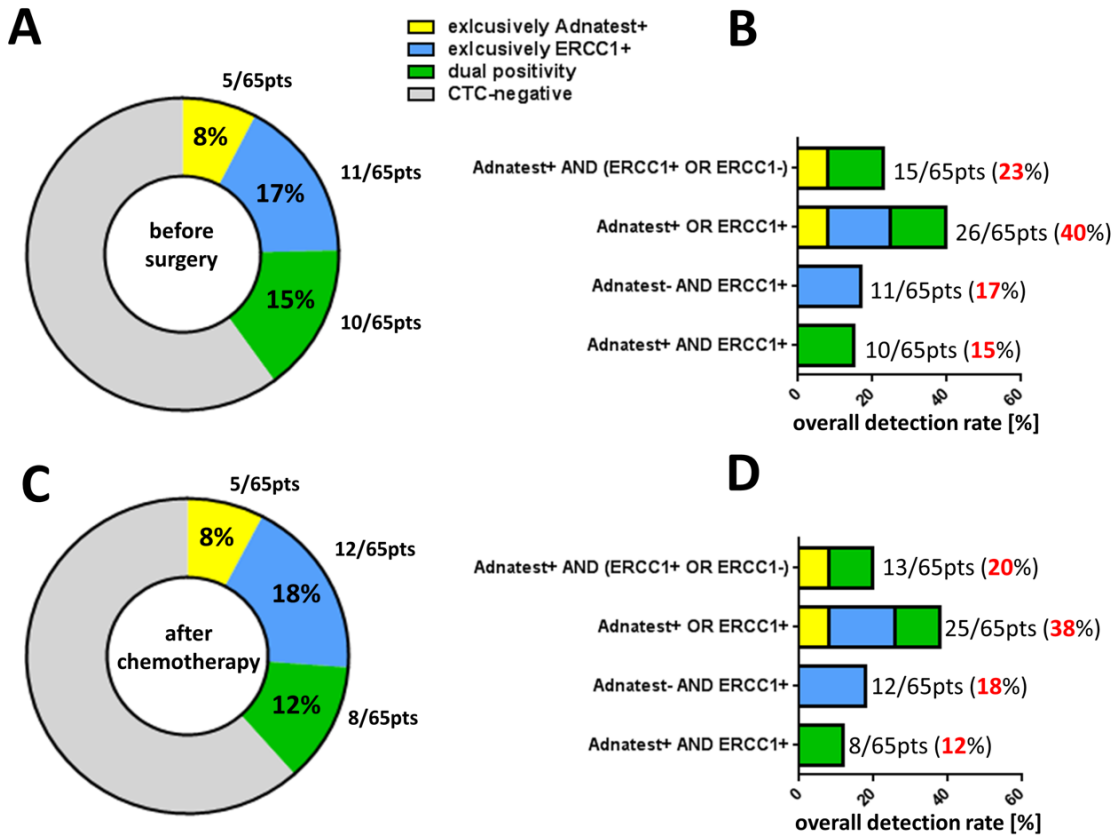


Abb.4.4 Schematische Übersicht der Distribution von ZTZ vor Therapie (A) und nach Therapie (C)

Die Kreisdiagramme zeigen die verschiedenen ZTZ-Phänotypen und ihre relativen Proportionen unter den untersuchten Ovarialkarzinompatientinnen vor der Operation und nach Therapie. Prozentsätze geben den Anteil der Patientinnen mit der exklusiven AdnaTest-Positivität (gelb), exklusiven ERCC1-Positivität (blau), der dualen Positivität für AdnaTest/ERCC1 (grün) und der ZTZ-negativen Patientinnen (grau) an. Die Balkendiagramme B (vor Therapie) und D (nach Therapie) fassen vier ZTZ-Kriterien zusammen, wobei die Expression von ERCC1 berücksichtigt wurde (Chebouti et al., 2016).

DNA-Platin-Addukte zu lösen und somit die Cisplatinvermittelte Zytotoxität und möglicherweise die Umwandlung in einen molekularen Phänotyp der „on target“ Platinresistenz zu umgehen (Galluzzi et al., 2012). Diese Annahme wird durch eine kürzlich erschienene Arbeit unterstützt, die die Anwesenheit von DNA-Platin-Addukten, Pt-(GpG) in ZTZ von fortgeschrittenen, nichtkleinzelligen Bronchialkarzinompatienten analysierte und das Fazit zog, dass PT-(GpG) Addukte in prä- und posttherapeutischen Blutproben für die Vorhersage und die Dosisindividualisierung der platinbasierten Chemotherapie ein potentieller Biomarker sein könnte (Nel et al., 2013). Ferner können $ERCC1_{pos}$ ZTZ mehrere Zyklen der Chemotherapie überleben und unter Berücksichtigung der Tatsache, dass ZTZ Metastasen initiieren können (Baccelli et al., 2013), könnten persistierende $ERCC1_{pos}$ ZTZ mit einem

platinresistenten Phänotyp das Potenzial haben, ein Rezidiv zu verursachen. Unter der Annahme, dass $ERCC1_{pos}$ ZTZ starke prognostische Faktoren in der posttherapeutischen Situation sind, insbesondere bei anhaltender Positivität, konnten unsere Daten auch zeigen, dass platinresistente $ERCC1_{pos}$ ZTZ direkt über die platinbasierte Chemotherapie selektiert werden können. Jedoch sind weitere funktionale Untersuchungen nötig, um diese Hypothese zu belegen. Unsere Studie ist aufgrund der begrenzten Anzahl an Patientinnen explorativ und hypothetisch. Dennoch markiert ERCC1 eine Subpopulation von ZTZ, die für die Überwachung der platinbasierten Chemotherapie und für die Beurteilung der therapeutischen Ergebnisse von Patientinnen mit Ovarialkarzinom nützlich sein könnte. Wir zeigen Grundlagen für die Validierung des klinischen Nutzens von $ERCC1_{pos}$ ZTZ in großen multizentrischen klinischen Studien und zur weiteren Aufklärung ihrer funktionalen und tumorbiologischen Bedeutung. Alternativ könnten Patientinnen mit $ERCC1_{pos}$ ZTZ von einer früh verabreichten und dosisintensiven Erhaltungstherapie mit Bevacizumab oder PARP-Inhibitoren profitieren, da einerseits die Versorgung der Tumorzellen verhindert wird und andererseits bei Zugabe eines PARP-Inhibitors die Reparaturmechanismen für Einzelstrangbrüche in der DNA der Tumorzelle ausgeschaltet werden, wodurch es leichter zu Doppelstrangbrüchen bei weiteren Zellteilungen kommt. Diese können in gesunden Zellen repariert werden, jedoch führt es in Tumorzellen mit BRCA1- und BRCA-2- Mutationen zur Apoptose (Fong et al., 2010; Chen et al., 2011).

Zusammenfassende Schlussfolgerung für die unter der Zielsetzung 1 und 2 erhobenen Daten:

Aus den Tumorzellanalysen hat sich herauskristallisiert, dass sowohl vor und nach Therapie DTZ mit Stammzellcharakter, als auch resistente ZTZ für die schlechte Prognose verantwortlich sein könnten. Des Weiteren zeigte sich unter Therapie, dass ZTZ mit mesenchymalem Charakter dominieren und $PI3K\alpha_{pos}/Twist_{pos}$ ZTZ nach Therapie als „neuer“ ZTZ Phänotyp präsent sind.

Unter Berücksichtigung all dieser Überlegungen werden nach Abschluss der Standardtherapien zusätzliche therapeutische Strategien benötigt, um diese ZTZ zu eliminieren. Möglich erscheint der Einsatz von mTOR-Inhibitoren, die „downstream“ von PI3K/AKT Signalwegen ansetzen (Zhang et al., 2011), Salinomyzin (Gupta et al.,

2009) oder neue synthetische Kurkumine gegen ALDH1 und GSK-3ß (Kesharwarni et al., 2015).

$ERCC1_{pos}$ ZTZ stellen eine für das Monitoring der Platintherapie wichtige Zellpopulation dar, um das Therapieansprechen einzuschätzen. Weiterhin könnten diese Patientinnen von einer früh initiierten und dosisintensivierten Erhaltungstherapie mit Bevacizumab oder PARP-Inhibitoren profitieren. Ferner könnte diese Hochrisikogruppe für zukünftige platinsensibilisierende Therapiestudien geeignet sein, die bereits präklinische und (Peng et al., 2010; Jazaeri et al., 2013; und Fukushima et al., 2014) klinisch (NCT01164995) bereits angeboten werden.

Die funktionelle Rolle von mesenchymalen bzw. semi-mesenchymalen ZTZ in der malignen Progression vom Ovarialkarzinom bedarf weiterer Analysen, um festzustellen, ob die EMT-ähnlichen ZTZ als Biomarker für ein Rezidiv beim Ovarialkarzinom verantwortlich sein könnten. In diesem Zusammenhang könnte ein erweitertes Multi-Marker-Gen-Panel, für das metastasierte Mammakarzinom bereits in der Frauenklinik in Essen etabliert (Bredemeier et al., 2016), einen umfangreicheren Aufschluss über ZTZ-Phänotypen im Hinblick auf eine zielgerichtete Therapie geben. Darüber hinaus könnten $PI3K\alpha_{pos}$ und $Twist_{pos}$ ZTZ ein attraktives therapeutisches Ziel sein, da PI3K/Akt/mTOR-Signalweginhibitoren derzeit für das Ovarialkarzinom in präklinischen und klinischen Studien untersucht werden (Liu et al., 2009; NCT01623349, NCT02476955).

Zielsetzung 3:

Untersuchungen zu small RNA Profilen

Zur Evaluierung von small RNA Profilen sollte, in Kooperation mit Herrn Dr. Kuhlmann aus der Frauenklinik in Dresden und Herrn Dr. Reuter aus dem Fraunhofer Institut in Leipzig, im Plasma von Patientinnen mit Ovarialkarzinom vor Therapie zunächst eine Methode etabliert werden, die mittels NGS das Profil je 15 platinsensitiver und platinresistenter Patientinnen darstellt, sodass im Anschluss ausgewählte small RNA Kandidaten als potentielle prädiktive Biomarker durch RT-qPCR validiert werden können.

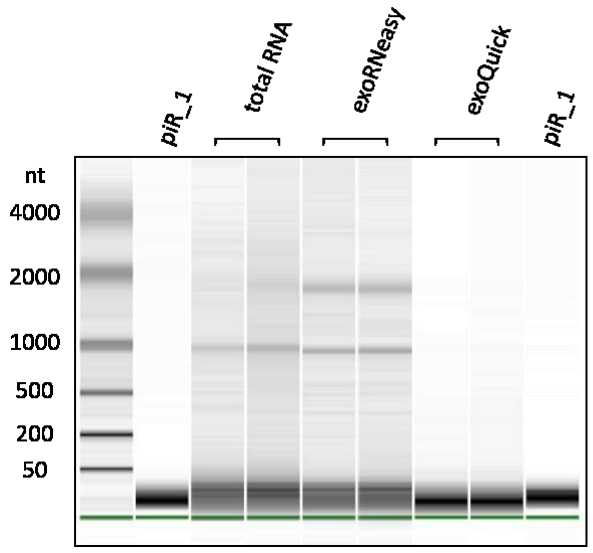
Die Experimente zu dieser Fragestellung sind abgeschlossen und die Ergebnisse werden in Kürze zu einer Publikation zusammengefasst. Im Folgenden werden der

„Arbeitsablauf“ und die wesentlichen Resultate kurz beschrieben.
Die Analyse der platinsensitiven und platinresistenten Gruppen zeigte, dass 12 differentiell exprimierte miRNA Kandidaten detektiert wurden. Jedoch zeigte sich hier keine Signifikanz. Darüber hinaus wurden 102 potentiell neue miRNAs detektiert, von denen 22 mindestens in der Hälfte der Proben über ein Minimum von zwei reads exprimiert wurden. Die Korrelationen der bekannten miRNA-Signaturen mit den klinischpathologischen Parametern ergaben keine signifikanten Korrelationen.

Vergleich von unterschiedlichen Isolationsmethoden für eine optimale RNA Präparation aus Plasma

Bei der Verwendung von Plasmaproben von gesunden Spendern verglichen wir die RNA-Mengen, generiert durch drei verschiedene Extraktionsmethoden, basierend auf der Isolierung von RNA aus 450µl Plasma oder aus vorangereicherten Mikrovesikeln. Verwendet wurden die Kits exoRNeasy und exoQuick der Firma QIAGEN, Hilden, Deutschland sowie eine Extraktionsmethode aus unfraktioniertem Plasma. Die RNA-Extraktion aus dem gesamten unfraktionierten Plasma führte zu einer Gesamtmenge von 18ng RNA. Die elektrophoretische Analyse zeigte eine relativ „unscharfe“ Größenverteilung zwischen 10 und 25nt. Unter Verwendung des exoRNeasy-Kits wurden nur etwa 13ng mit einer vergleichbaren Größenverteilung gewonnen. Eine optimale, mehr als 3-fach erhöhte, RNA-Menge wurde mit exoQuick (~60ng) mit einer stärker fokussierten und deutlicheren Größenverteilung um 20nt erhalten (Abb.4.5). Aufgrund dieser Ergebnisse wählten wir als Voranreicherung die auf Mikrovesikeln basierende Methode unter Verwendung von exoQuick als Methode der Wahl.

Vor der Kartierung wurden die „reads“ auf mögliche technische Artefakte und biologische Verunreinigungen untersucht. Plasmaproben zeigten in beiden Kategorien keine Anzeichen. Es wurden keine verbleibenden Adapter nach dem Ausschnitt gefunden. Die Ausrichtung auf bakterielle, virale, menschliche ribosomale, menschliche Globin-, Größenauswahl-Oligo- und Klonierungsvektoren Referenz-sequenzen ergaben vernachlässigbare Mengen an Überlappungen. Die Anwesenheit von miRNAs im Patientenplasma wurde als der Anteil der „reads“ pro miRNA beschrieben.

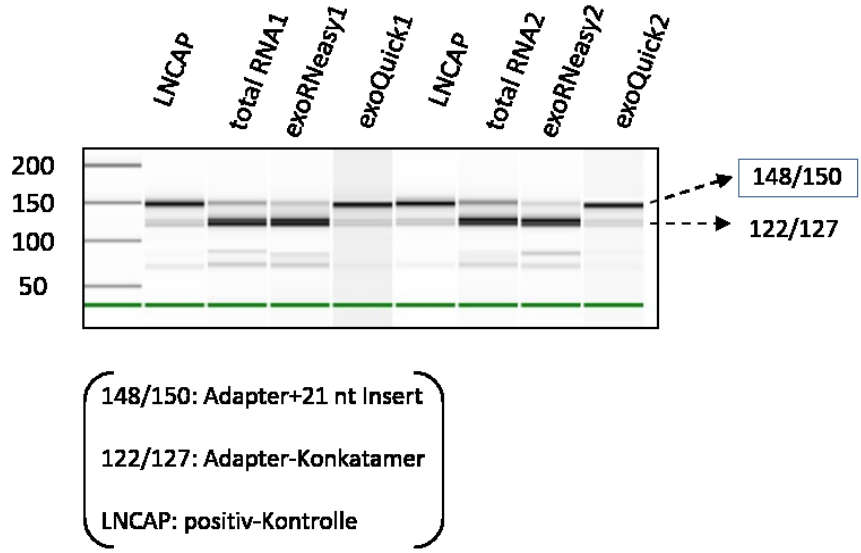


Probe	RNA [ng/µl]	gesamt [ng]
total RNA_1	1,3	18,1
total RNA_2	1,3	18,9
exoRNeasy_1	1,0	13,7
exoRNesay_2	0,9	12,8
exoQuick_1	4,3	60,0
exoQuick_2	4,4	61,8

Abb.4.5 Vergleich der gewonnen RNA-Mengen aus Plasma mittels verschiedener Extraktionsmethoden (Totale RNA, exoRNeasy und exoQuick)

Vergleichende "Library Preparation" mit small RNA aus unterschiedlichen Isolationsmethoden

RNA-Proben aus den verschiedenen oben beschriebenen Isolationsverfahren wurden einer Größenfraktionierung, unter Verwendung von Polyacrylamidgelen, unterworfen. Die 20nt kleine RNA-Fraktion, die miRNA und andere kleine RNA-Spezies enthielt, wurde isoliert und extrahiert. Anschließend wurden kleine RNAs kloniert, um eine kleine „RNA-Library" für smallRNA-Sequenzierung zu präparieren. Die folgende Abbildung gibt einen Überblick über die daraus resultierenden PCR-Produkte aus den verschiedenen RNA-Proben nach Klonierung (Abb.4.6) wieder. Das 148/150 bp Amplicon stammt aus dem amplifizierten 21 nt Insert bzw. der Adaptersequenz, während das 122/127bp Amplicon die Adapter bzw. die Konkatamersequenz darstellt. RNA-Proben, die aus der exoQuick-Präparation gewonnen wurden, erzeugten die höchste molare Konzentration von PCR-Produkten im Vergleich zu den exoRNeasy-Proben und der unfraktionierten Gesamt-RNA, ähnlich der Positivkontrolle aus LNCAP Zellen.



Probe	~ 150 nt PCR (nM)
total RNA_1	4,4
total RNA_2	5,9
exoRNeasy_1	2,3
exoRNesay_2	3
exoQuick_1	57
exoQuick_2	74

Abb.4.6 Vergleich der Library preparation aus zirkulierender smallRNA

Bioinformatik

Für die bioinformatische Analyse wurde eine ungefähre Sequenztiefe von drei Millionen reads pro Probe gewählt, um sicherzustellen, dass bekannte miRNAs, die eine Sequenztiefe von etwa zwei Millionen reads bedürfen, detektiert werden. Die RNA-Sequenzierungsanalyse ergab durchschnittlich 3,4 Millionen „reads“ (min: 2,5, max 5,7) pro Probe. Kontrollen und Plasmaproben zeigten einen Hauptpeak um 22nt und stehen im Einklang mit den miRNA-Sequenzen, ohne Anzeichen für andere smallRNA-Klassen wie piRNA (29nt), tRNA (30nt) und YRNA (32nt). Die Daten zur Analyse der Komplexitäten sind hier nicht dargestellt.

Ergebnisse

Die Analyse zeigte, das 10 schon bekannte miRNAs detektiert wurden die am stärksten in allen Proben exprimierten (hsa-miR-128-3p, hsa-miR-99a-5p, hsa-let-7i-5p, hsa-miR-148a-3p, hsa-miR-129-5p, hsa-miR -381-3p, hsa-miR-9-3p, hsa-miR-9-5p, hsa-miR-433-3p, hsa-let-7b-5p). Die Korrelationen der bekannten miRNA-Signaturen mit den klinischpathologischen Parametern ergaben keine signifikanten Korrelationen.

Die differentielle miRNA-Expressionsanalyse wurde mit DESeq2 an reifen und Vorläufer-miRNA durchgeführt. Die folgende Abbildung zeigt eine Auswahl von 12 maturen miRNA Sequenzen, die die niedrigsten p-Werte zeigten und differentiell in Plasmaproben von platinsensitiven vs. platinresistenten Ovarialkarzinompatientinnen

exprimiert wurden. Nach der Adjustierung der p-Werte erreichten diese Befunde jedoch keine statistische Signifikanz (Abb.4.7).

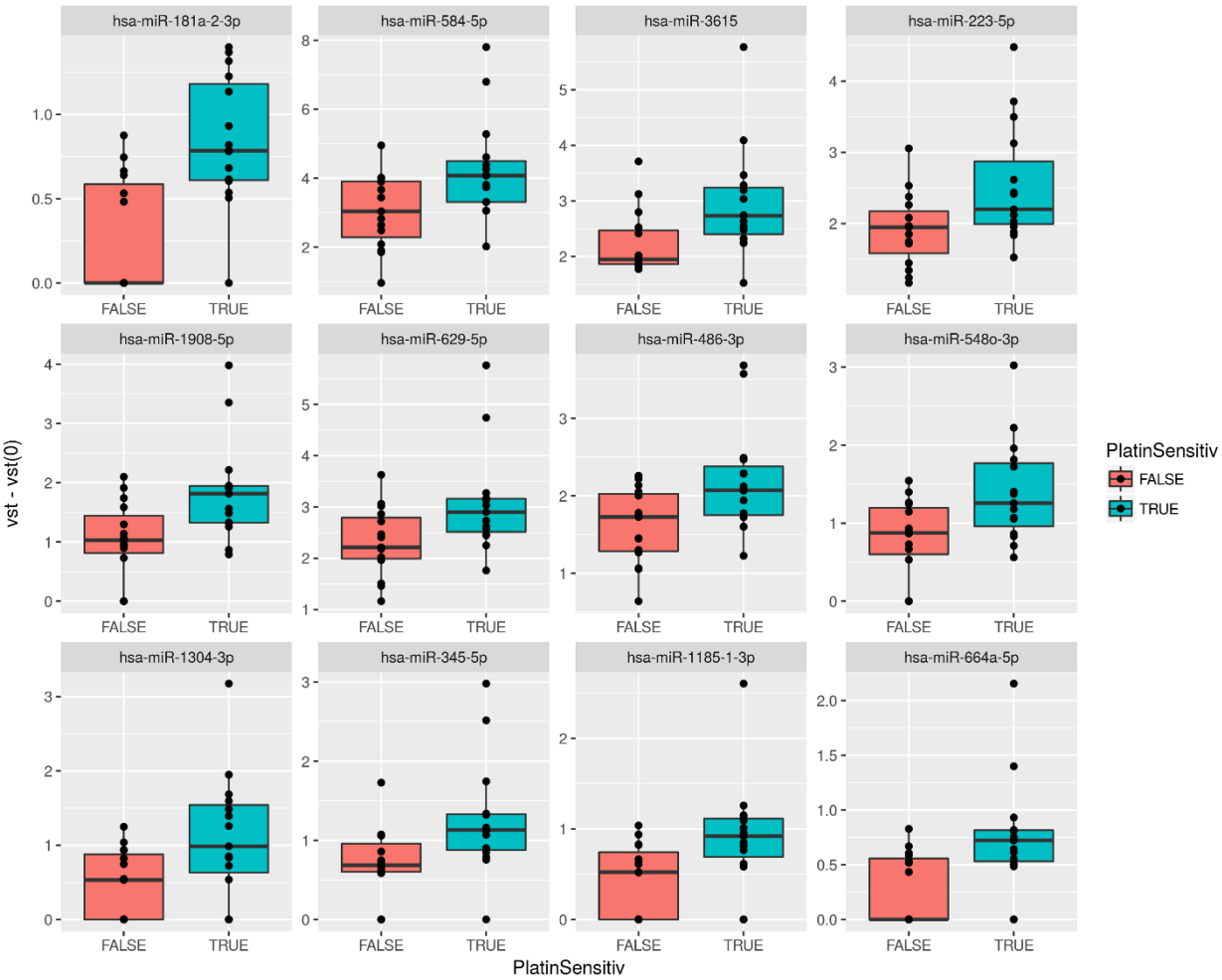


Abb.4.7 Differentielle Häufigkeit von reifen zirmiRNA im Plasma von platinsensitiven vs. platinresistenten Ovarialkarzinompatientinnen

Anschließend wurde eine differentielle miRNA-Expressionsanalyse für Vorläufer-miRNA-Sequenzen durchgeführt. Die Abb.4.8 zeigt eine Auswahl von 12 precursor miRNA Sequenzen, die die niedrigsten p-Werte zeigten und differentiell in Plasmaproben von platinsensitiven vs. platinresistenten Ovarialkarzinompatientinnen exprimiert wurden. Auch hier, wurde nach der Anpassung der p-Werte keine statistische Signifikanz erreicht.

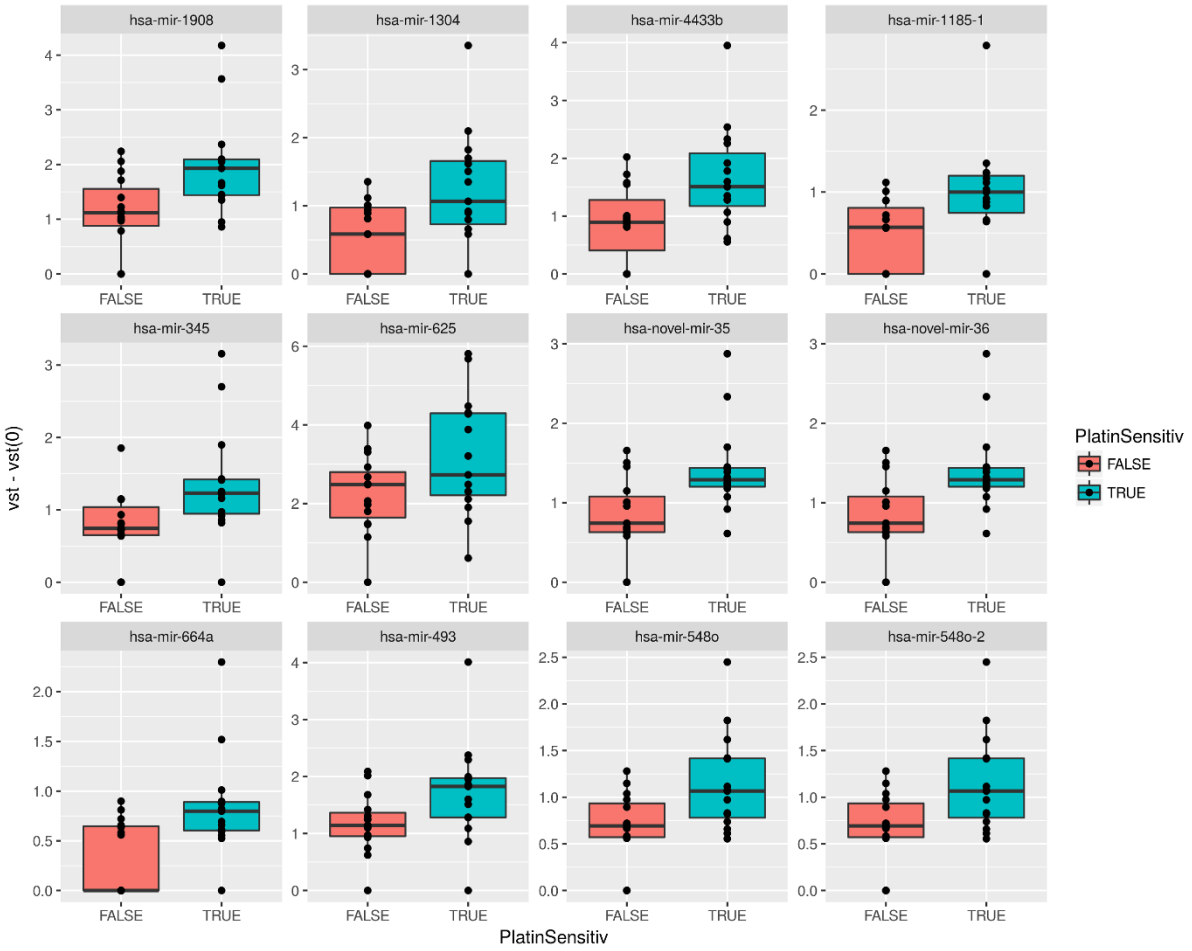


Abb.4.8 Differentielle Häufigkeit von Vorläufer-zirmiRNA im Plasma von platinsensitiven vs. platinresistenten Ovarialkarzinompatientinnen

Die angewandte-miRNA-Detektionssoftware erkennt durch die Ausrichtung auf das menschliche Genom und die sekundäre Strukturvorhersage potenziell neuartige miRNAs. Für die hier untersuchten Plasmaproben wurden insgesamt 102 neue miRNAs nachgewiesen, von denen 22 mindestens in der Hälfte der Proben über ein Minimum von zwei „reads“ exprimiert wurden.

Schlussfolgerung:

Im Rahmen der hier beschriebenen Untersuchungen wurde ein angepasster Workflow für die Aufreinigung zirkulierender small RNAs aus humanen Plasmaproben mit anschließender NGS-basierte Analyse optimiert. Dieser Workflow wurde anschließend exemplarisch an Hand einer klinisch-translationalen Fragestellung getestet, in dem das zirkulierende miRNA Profil im Plasma von platinsensitiven vs. platinresistenten Ovarialkarzinompatientinnen verglichen wurde. Tendenziell zeigte sich hier eine präliminäre Auswahl von differentiell exprimierten miRNA Kandidaten, die Ergebnisse erreichten jedoch im vorliegenden Pilotkollektiv (noch) keine statistische Signifikanz. Weitere Untersuchungen in unabhängigen Validierungskohorten werden zeigen,

inwieweit diese miRNA Kandidaten als potentiell prädiktive Biomarker für die Platinresistenz des Ovarialkarzinoms genutzt werden können.

Nebenziel: RASSF-1A Promotormethylierung

Die Ergebnisse dieser Studie wurden in Oncotarget publiziert.

RASSF1A promoter methylation in high-grade serous ovarian cancer: A direct comparison study in primary tumors, adjacent morphologically tumor cell-free tissues and paired circulating tumor DNA.
Lydia Giannopoulou, Issam Chebouti, Kitty Pavlakis, Sabine Kasimir-Bauer, Evi Lianidou. Oncotarget 2017*. doi: 10.18632/oncotarget.15249.*

Obwohl es schon Studien zur RASSF-1A Promotormethylierung im Tumorgewebe von Ovarialkarzinompatientinnen gibt, analysierte unsere Studie zum ersten Mal den Methylierungsstatus von RASSF-1A im Tumorgewebe, dem Normalgewebe und den entsprechenden korrespondieren Plasmaproben.

In Kooperation mit der Arbeitsgruppe von Frau Prof. Lianidou der Universität Athen wurde die RASSF1A Promotermethylierung im Tumorgewebe (n=61), im angrenzenden tumorfreien Gewebe (n=58) und in korrespondierenden Plasmaproben (n=59) von Patientinnen mit primärem Ovarialkarzinom, mittels der MSP-PCR und der semiquantitativen „methylation-sensitive high-resolution melting analysis“ (MS-HRMA) untersucht. Die Frauenklinik stellte hier die unabhängige Validierungskohorte mit 61 Gewebetumorproben, 58 angrenzenden tumorfreien Geweben und 59 korrespondierenden Plasmaproben zur Verfügung. Die schematische Überschrift der Studie zeigt zur Veranschaulichung die experimentelle Vorgehensweise (Abb.4.9). Die Isolierung der zirfDNA aus dem Plasma, die Sodiumbisulfitkonvertierung und die MSP-PCR sowie MS-HRMA wurden in Athen durchgeführt. Die MSP-PCR ergab eine RASSF1A-Methylierung in 25/61 (41%) Tumorproben, in 17/58 (29%) gesunden Geweben und in 15/59 (25%) Plasmaproben. Im Gegensatz dazu wurde bei der MS-HRMA-Analyse eine Methylierung des Gens im Tumorgewebe in 28/61 (46%) und im gesunden Gewebe in 21/58 (36%) der Patientinnen detektiert. Die Methylierung des Gens im Plasma wurde mit dieser Methode nicht untersucht. Die RASSF-1A-Promotor-Methylierung assoziierte signifikant mit der Tumorqualität, unter Verwendung beider Assays, sowie mit dem Befall regionaler Lymphknoten unter Verwendung von MS-HRMA. Durch MS HRMA-Analysen im Tumorgewebe wurde gezeigt, dass der RASSF-1A-Promotor-Methylierungsstatus signifikant mit dem verkürzten OS korrelierte. Im Gegensatz dazu wurde unter Verwendung der Real-Time-MSP Analyse keine signifikante Korrelation mit dem PFS und OS beobachtet.

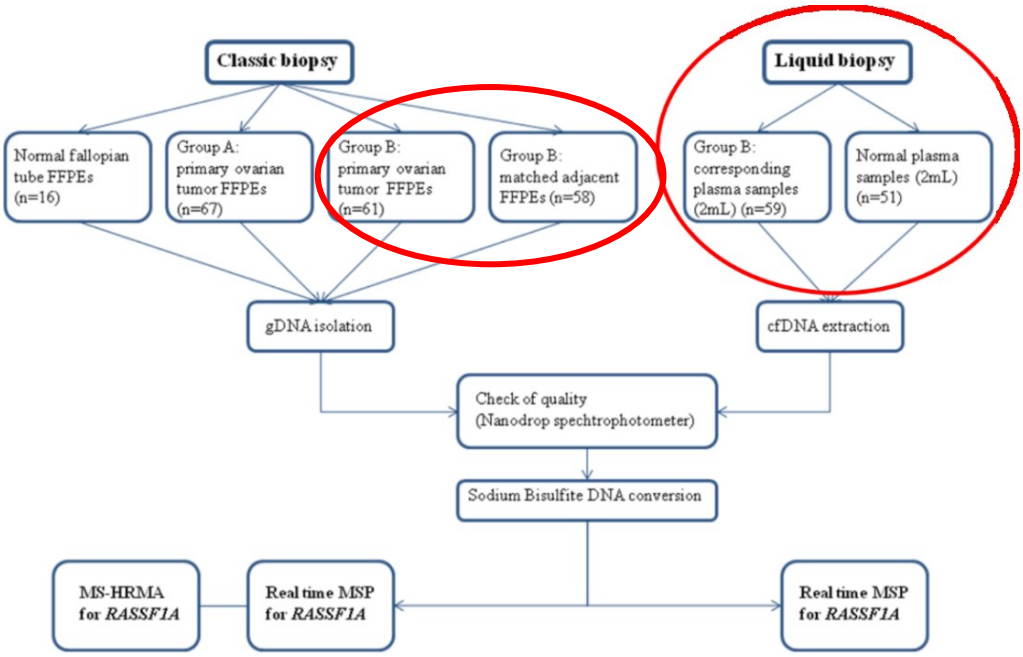


Abb.4.9 Schematische Übersicht der RASSF-1A Studie.
Das rot eingekreiste Feld stellt die Validierungskohorte der Frauenklinik Essen dar **(Giannopoulou et al., 2017).**

In dieser Studie konnte zum ersten Mal gezeigt werden, dass der RASSF-1A-Promotor im benachbarten tumorfreien Gewebe methyliert ist und dass die RASSF-1A-Promotor-Methylierung prognostische Informationen hat. Unter Verwendung von MS-HRMA (36,2%) und Real-Time-MSP (29,3%) beobachteten wir ein sehr hohes Methylierungsniveau. Der Unterschied könnte in dem Feldeffekt begründet sein. Dieser beschreibt einen biologischen Prozess, bei dem große Zellbereiche an einer Gewebeoberfläche oder innerhalb eines Organs von einer karzinogenen Veränderung betroffen sind. Der Prozess entsteht aus der Exposition gegenüber einer schädlichen Umgebung, oft über einen längeren Zeitraum (Heaphy et al., 2009; Torezan et al., 2013). Es fanden sich keine signifikanten Unterschiede zwischen dem Methylierungsniveau vom Tumor und dem angrenzenden gesunden Gewebe. In drei Fällen war der Prozentsatz der RASSF-1A-Promotor-Methylierung im benachbarten Gewebe höher als im Tumor. Eine mögliche Erklärung ist der starke Feldeffekt, der die untersuchten Fälle kennzeichnet. Nach unseren Erkenntnissen korrelierte die RASSF-1A-Promotor-Methylierung signifikant mit dem OS, wenn MS-HRMA verwendet wurde. Es konnte allerdings keine signifikante Korrelation mit der Real-Time-MSP festgestellt werden. Diese Tatsache gibt der MS-HRMA-Methode einen Vorteil, obwohl die Real-Time-MSP-Methode ein sensibler Assay ist. Allerdings wird die Real-Time-MSP für Methylierungsstudien in Plasma-ztDNA aufgrund ihrer höheren Sensitivität bevorzugt.

In sechs Proben, bei denen der Primärtumor unmethyliert gefunden wurde, waren die entsprechenden Plasmaproben methyliert. Eine mögliche Erklärung ist die Tumorheterogenität. Es ist klar, dass die Gewebebiopsie eine Momentaufnahme des Oberflächenprofils eines Tumors darstellt, während die zfDNA die gesamten genetischen und epigenetischen Eigenschaften eines bestimmten Tumors widerspiegelt.

Zusammenfassende Schlussfolgerung für die unter Zielsetzung 3 und das Nebenziel erhobenen Daten:

Als weitere, nicht zelluläre, „Liquid Biopsy“ sind zirkulierende DNA und miRNA attraktive Marker für das Ovarialkarzinom. Bezüglich der miRNAs konnten potentielle Kandidaten zur weiteren Evaluierung identifiziert werden, um platinresistente und platinsensible Patientinnen zu beschreiben. Die Ergebnisse des RASSF-1A-Methylierungsstatuses beim Ovarialkarzinom haben das Potenzial, wichtige prognostische Informationen zu geben. Um diese Art der „Liquid Biopsy“ zukünftig zu nutzen, müssen prospektive Studien durchgeführt werden. Weitere Untersuchungen in unabhängigen Validierungskohorten werden zeigen, inwieweit diese Kandidaten als potentielle prädiktive Biomarker für das Ovarialkarzinom, speziell für die Vorhersage der Platinresistenz, in der Klinik eingesetzt werden können.

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6 Anhang

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6.2 Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

6.3 Eidesstattliche Erklärung

Erklärung

Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

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Erklärung

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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